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FOREWORD

It has been decided to change the title of the Australian Journal of Scientific Research, Series B: Biological Sciences, to the Australian Journal of Biological Sciences commencing with this issue. The volume numbering continues that of the earlier Journal.

THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

IV. SEASONAL VARIATION IN CELL SIZE, NITROGEN METABOLISM, AND RESPIRATION IN DEVELOPING GRANNY SMITH APPLE FRUITS

By Judith A. Pearson[*] and R. N. Robertson[*]

[*Manuscript received August* 11, 1952]

Summary

Results of investigation of cell size, fruit size, nitrogen metabolism, and respiration rate over three successive seasons confirm the main conclusions of earlier papers in this series. Fruit left on the trees for a period beyond normal commercial maturity showed a very large increase in soluble nitrogen with only slight increase in protein nitrogen. The relation of the nitrogen and organic acid metabolism to the climacteric rise in respiration is discussed.

I. Introduction

Earlier papers in this series recorded observations on the physiology of the Granny Smith apple fruit, with particular reference to cell enlargement (Bain and Robertson 1951; Robertson and Turner 1951). This paper reports the investigation of fruits developing on the same trees in three later consecutive seasons. Further, by an improved sampling technique and by continuing the sampling over a longer period, a more detailed study of several aspects was possible.

In two complete seasons, October to June of 1949-50 and 1950-51, and one part season, February to June of 1949, the anatomical and chemical changes occurring during the development of the fruits were investigated. Analysis included determination of cell size, cell number, respiration rate, total and protein nitrogen, total organic acids, starch, and cell wall material. Ninety-two fruits from 10 samples in the 1949-50 season were examined individually for each of these factors.

An attempt has been made to relate respiration rate and changes in chemical composition to cell size changes, and to discuss the metabolic processes associated with the climacteric rise in respiration.

II. Material and Methods

(*a*) *Sampling*

In the three seasons samples of Granny Smith apples were taken from two trees at Bathurst used in the earlier work. Most of the results described were from one tree in the two complete seasons; results from the other tree are also discussed where there is any appreciable difference. Samples were taken every 3 weeks from two trees in 1949-50 and monthly from one tree only in 1950-51.

---

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In 1949-50 one tree had approximately 500 fruits at 40 days from full blossom and the other had approximately 560; in 1950-51, at 54 days from full blossom, the numbers were 250 and 180 respectively. Picking dates and times from full blossom are shown in Table 1.

Table 1

DATE OF SAMPLING SEASON

Days from Full Blossom	1948-49		1949-50		1950-51	
	Pick	Date	Pick	Date	Pick	Date
0	—	—	1	6.x.49	—	—
40	—	—	2	15.xi.49	—	—
54	—	—	—	—	1	26.xi.50
59	—	—	3	4.xii.49	—	—
74	—	—	4	19.xii.49	—	—
76	—	—	—	—	2	18.xii.50
102	—	—	5	16.i. 50	—	—
104	—	—	—	—	3	15.i.51
123	—	—	6	6.ii.50	—	—
129	1	9.ii.49	—	—	—	—
132	—	—	—	—	4	12.ii.51
144	—	—	7	27.ii.50	—	—
155	2	7.iii.49	—	—	—	—
160	—	—	—	—	5	12.iii.51
165	—	—	8	20.iii.50	—	—
169	3	22.iii.49	—	—	—	—
183	4	5.iv.49	—	—	—	—
188	—	—	—	—	6	9.iv.51
193	—	—	9	17.iv.50	—	—
198	5	20.iv.49	—	—	—	—
207	—	—	10	1.v.50	—	—
211	6	3.v.49	—	—	—	—
216	—	—	—	—	7	7.v.51
225	7	19.v.49	—	—	—	—
228	—	—	11	22.v.50	—	—
239	8	30.v.49	—	—	—	—
242	—	—	12	5.vi.50	—	—
244	—	—	—	—	8	4.vi.51
263	—	—	13	26.vi.50	—	—
277	—	—	14	10.vii.50	—	—

In 1948-49 each sample consisted of 15 fruits. To improve the sampling in subsequent seasons, each tree was divided into 20 sections, with roughly equal numbers of fruits at the beginning of the season. Each pick contained one fruit, taken at random, from each section. The fruits of each sample were bulked for analysis. In the 1949-50 season five fruits from picks 5-14 were kept as individuals for all the analyses and the remaining 15 were treated in bulk.

(*b*) *Respiration Rate*

Respiration rate was measured by the Pettenkofer method at 25°C., as described by Turner (1949). The results are given as equivalent rates at 20°C. assuming a Q_{10} of 2. Individual respiration rates were measured by enclosing each fruit in a glass shell; the bulk samples were enclosed in large vacuum desiccators.

(*c*) *Cutting and Drying*

After the respiration measurements the fruits were cut transversely and four thin transverse sections were taken from one half on two diameters at right angles. The sections were kept in formalin : alcohol (60 ml. : 1000 ml. 70 per cent.) for anatomical study.

After the sections for the measurement of cell size had been removed, the fruits were peeled and the cortical tissue was sliced thinly. For moisture estimation one-quarter of each fruit was grated, and an aliquot of the carefully mixed material dried at 70°C. in a weighed aluminium can, first in an air-draught oven for several hours and then in a vacuum oven for 6 hr. Material for analysis was dried in the same way, cooled, ground finely, and stored in air-tight jars.

(*d*) *Analytical*

The analytical methods used were those described by Turner (1949) except for the estimation of total organic acids. In the method used, 0.5-1.0 g. dried apple powder was taken up in about 200 ml. distilled water. This was titrated with 0.1N sodium hydroxide, using phenolphthalein as indicator, to determine free acids. A further 0.5-1.0 g. dried apple powder was weighed in a porcelain basin and ashed at a low temperature to avoid loss of potassium. It was heated on a gauze mat while the fumes were given off and later on a triangle for about 10 min. until the ash was a light grey colour. After cooling, 5 ml. 0.1N hydrochloric acid were added to the ash and allowed to stand for 5 min. This was diluted to approximately 200 ml. and boiled to expel carbon dioxide. It was cooled immediately and back-titrated with 0.1N sodium hydroxide to determine the equivalents of combined acid. The free and combined acids together made up the total acids. The results of some analyses by this method were compared with the 48-hr. ether-extraction method of Pucher, Wakeman, and Vickery (1941). This gave a slightly higher result but the difference did not justify the extra work involved in using the ether method.

III. RESULTS

(*a*) *Anatomical*

(i) *Cell Size.*—As in the earlier work (Bain and Robertson 1951), 25 cells from two transverse sections of the cortex across the equator of the fruit were traced and measured to calculate the average cell volume. The results for tree 2 in three seasons are set out in Table 2.

Changes in cell volume in both 1949-50 and 1950-51 seasons followed closely the changes in fruit weight with time (Figs. 1 and 2). Both fruit weight and cell volume in the 1950-51 season were very much lower than in the 1949-50 season. From an examination of the individual fruits in the 1949-50 samples, cells from the small and large fruits within a pick had much the same cell volume, so that cells in both were growing at the same rate. Cell volume increased throughout the season but at a lower rate after about 190 days from full blossom when the fruits were commercially mature.

TABLE 2

CHANGES IN MEAN CELL VOLUME AND MEAN FRUIT WEIGHT WITH TIME FROM FULL BLOSSOM

Days from Full Blossom	1948-49		1949-50		1950-51	
	Fruit Weight (g.)	Cell Volume (cu. mm.)	Fruit Weight (g.)	Cell Volume (cu. mm.)	Fruit Weight (g.)	Cell Volume (cu. mm.)
0	—	—	0·106	—	—	—
40	—	—	7·75	0·0003	—	—
54	—	—	—	—	13·95	0·0004
59	—	—	24·73	0·0007	—	—
74	—	—	42·97	0·0015	—	—
76	—	—	—	—	27·79	0·0007
102	—	—	81·85	0·0024	—	—
104	—	—	—	—	66·05	0·0020
123	—	—	123·84	0·0024	—	—
129	116·29	0·0038	—	—	—	—
132	—	—	—	—	98·97	0·0030
144	—	—	149·99	0·0038	—	—
155	153·67	0·0051	—	—	—	—
160	—	—	—	—	132·12	0·0035
165	—	—	192·84	0·0047	—	—
169	179·13	0·0056	—	—	—	—
183	197·13	0·0059	—	—	—	—
188	—	—	—	—	170·28	0·0039
193	—	—	250·01	0·0056	—	—
198	195·75	0·0059	—	—	—	—
207	—	—	246·38	0·0055	—	—
211	202·91	0·0064	—	—	—	—
216	—	—	—	—	193·01	0·0042
225	217·01	0·0062	—	—	—	—
228	—	—	245·88	0·0058	—	—
239	222·08	0·0061	—	—	—	—
242	—	—	296·51	0·0058	—	—
244	—	—	—	—	182·78	0·0046
263	—	—	267·25	0·0063	—	—
277	—	—	257·60	0·0060	—	—

In the 1948-49 season the fruit development was different. Cell volume was larger than in either of the other seasons but the fruit weight was less than in the 1949-50 season.

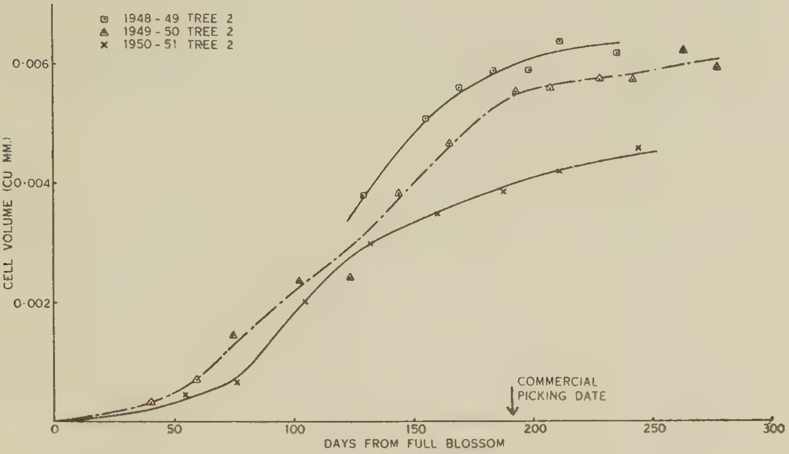

Fig. 1.—Changes in cell volume with time.

(ii) *Cell Number*.—Cell number was determined by the method of Bain and Robertson (1951), and the results for the three seasons are given in Table 3. Cell volume varied little within a pick but cell number ranged from about 12,000,000 to 83,000,000 with an average in the later picks of about 40,000,000. This accounted for the difference in fruit weight in fruit of the same age and agrees with earlier work.

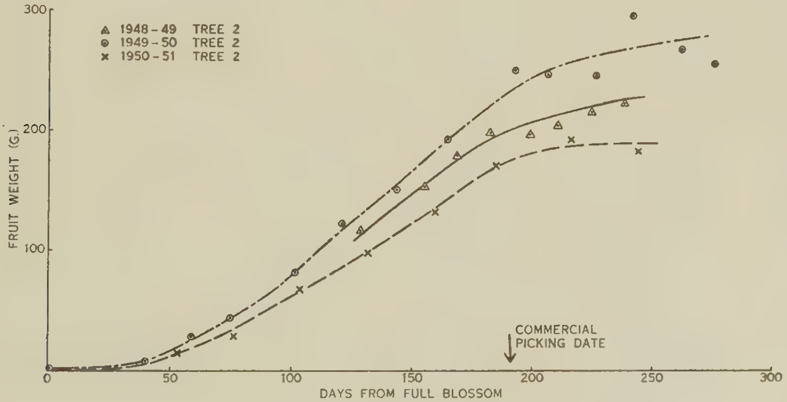

Fig. 2.—Changes in fruit weight with time.

In the 1950-51 season, additional samples were taken at full blossom, at 3 weeks, and at 6 weeks from full blossom. The results are shown in Table 4. The change in cell volume during the first 3 weeks was very small but the cell number increased about 10 times. By 6 weeks, the cell number was approximately equal to the mean cell number for the season, showing that cell division had almost ceased; cell volume had increased considerably in the second 3 weeks.

Mean cell number for the three seasons is plotted against time in Figure 3. In analysing these data it was found that the distribution of the number of cells within a pick was noticeably skew but the logarithm of the cell number was approximately normally distributed. Using the logarithmic transformation, the differences between the means of the picks were significant in relation to the variation between fruits within the picks. The cause of these differences between picks is not known. The cell number was significantly lower in 1948-49 than in the other two seasons.

TABLE 3

MEAN CELL NUMBER IN RELATION TO TIME

Days from Full Blossom	1948-49 Cell Number $\times 10^{-6}$	1949-50 Cell Number $\times 10^{-6}$	1950-51 Cell Number $\times 10^{-6}$
0	—	2·69	—
40	—	22·0	—
54	—	—	28·8
59	—	31·7	—
74	—	26·9	—
76	—	—	41·0
102	—	31·4	—
104	—	—	29·6
123	—	46·9	—
129	29·9	—	—
132	—	—	29·6
144	—	35·5	—
155	22·2	—	—
160	—	—	34·7
165	—	37·3	—
169	30·8	—	—
183	31·2	—	—
188	—	—	40·0
193	—	41·0	—
198	33·4	—	—
207	—	40·1	—
211	32·6	—	—
216	—	—	41·5
225	30·5	—	—
228	—	38·8	—
239	32·0	—	—
242	—	46·8	—
244	—	—	36·0
263	—	38·9	—
277	—	39·3	—

The earlier work of Tetley (1930, 1931) and of Bain and Robertson (1951) showed that cell division was completed in about 3 weeks from full blossom. The points in Figure 3 indicate a slight increase in cell number from 50 days to the end of the season. We do not believe that this necessarily contradicts the conclusions of the earlier work, in which direct observation failed to show cell division after about 3 weeks. The apparent increase in cell numbers shown in Figure 3 may arise from the method of calculating cell number from measurements of cells in the mid cortex; this method may not be equally satisfactory over the whole season for fruits of different sizes. We use the calculated mean cell numbers for expressing analytical results; any errors introduced by doing this will not affect the conclusions reached in this paper.

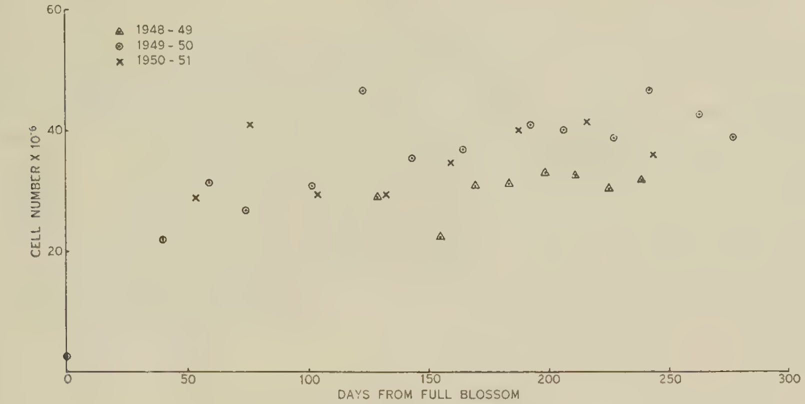

Fig. 3.—Changes in cell number with time.

TABLE 4

CHANGES IN CELL VOLUME AND CELL NUMBER DURING THE FIRST 7 WEEKS OF FRUIT GROWTH

Pick	Date	Tree 2		Tree 3	
		Mean Cell Volume (cu. mm.)	Mean Cell Number $\times 10^{-6}$	Mean Cell Volume (cu. mm.)	Mean Cell Number $\times 10^{-6}$
1	3.x.50	0·000036	1·6	0·000027	2·4
2	25.x.50	0·000038	20·5	0·000044	18·5
3	15.xi.50	0·000249	33·8	0·000259	35·5

(iii) *Fruit Size.*—The results of the three seasons confirm the observation given in the previous paper that variation in fruit size is determined early in development by the amount of cell division. Within one season the cell volume in individual fruits of the same age but of different size was fairly uniform so that the variation in fruit size was determined by cell number and, to some extent, by the amount of air space. Between seasons, however, the size of cells may be the overriding factor in determining fruit size. This is shown by comparison of the 1949-50 and the 1950-51 seasons when the fruits had similar cell numbers, but in the later season they had smaller cells and hence the fruit size was lower, possibly because of a dry period early in development.

(*b*) *Analytical*

(i) *Dry Weight.*—Dry weight as a percentage of fresh weight increased about 7-8 per cent. during the season. The dry weight per cell was proportional to cell volume, both in time and in the individual fruits of a pick. Increase in dry weight per cell with time in tree 2 is shown in Figure 4.

In 1949-50, fruits on tree 3 behaved differently from those on tree 2 for about a month after the date of commercial maturity and showed a loss of dry weight. This appeared to be a real check in the growth of the fruit and was reflected in a similar decrease in total and protein nitrogen, starch, and organic acids.

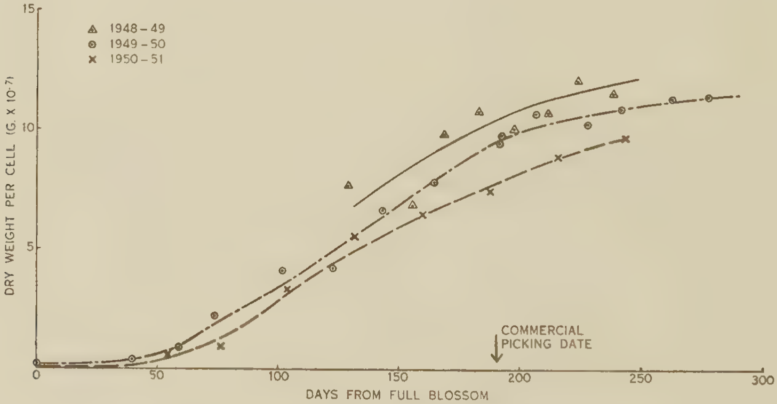

Fig. 4.—Changes in dry weight per cell with time.

(ii) *Starch.*—Starch was estimated on the dried material. A comparison with estimations on fresh material indicated that drying did not alter the results within the limits of experimental error. It is unlikely that any appreciable change in starch content occurred during the 72 hr. between picking and cutting. Kidd *et al.* (1950) found that synthesis of starch in Bramley's Seedling apples continued for 1-2 days after the fruits were removed from the tree; starch then began to decrease.

Starch was detected in the cells in measurable quantities at 60 days from full blossom in 1949-50 and at 76 days in 1950-51. Starch determinations on the individual fruits of each pick in 1949-50 showed the mean starch content per cell to be very variable between fruits. Starch per cell rose from about 0.2×10^{-8} g. at 60 days to 11.9×10^{-8} g. at 165 days from full blossom. It decreased again rapidly after this to 0.3×10^{-8} g. at 263 days. In 1950-51 starch per cell increased from 0.2×10^{-8} g. at 76 days to 5.9×10^{-8} g. at 160 days and dropped again to 0.3×10^{-8} g. at 244 days from full blossom (Table 5, Fig. 5).

The peak in starch synthesis occurred at the same time in all three seasons but in the latest season, when the cell volume and fruit weight were considerably lower, the amount of starch per cell was only half that formed in the earlier seasons.

Table 5

CHANGE IN STARCH AND CELL WALL MATERIAL PER CELL WITH TIME IN EACH SEASON

Days from Full Blossom	1948-49	1949-50		1950-51	
	Starch per Cell (g. $\times 10^{-8}$)	Starch per Cell (g. $\times 10^{-8}$)	Cell Wall Material per Cell (g. $\times 10^{-8}$)	Starch per Cell (g. $\times 10^{-8}$)	Cell Wall Material per Cell (g. $\times 10^{-8}$)
0	—	—	—	—	—
40	—	—	2·0	—	—
54	—	—	—	—	1·9
59	—	0·2	4·3	—	—
74	—	2·1	8·6	—	—
76	—	—	—	0·2	2·5
102	—	8·3	8·5	—	—
104	—	—	—	1·8	6·4
123	—	8·3	4·9	—	—
129	6·6	—	—	—	—
132	—	—	—	4·9	8·3
144	—	11·0	10·6	—	—
155	—	—	—	—	—
160	—	—	—	5·9	6·1
165	—	11·9	9·8	—	—
169	12·2	—	—	—	—
183	9·9	—	—	—	—
188	—	—	—	4·3	6·2
193	—	6·0	18·4	—	—
198	1·7	—	—	—	—
207	—	9·2	9·9	—	—
211	2·8	—	—	—	—
216	—	—	—	1·5	6·5
225	0·6	—	—	—	—
228	—	4·4	10·9	—	—
239	0·9	—	—	—	—
242	—	2·1	10·8	—	—
244	—	—	—	0·3	5·6
263	—	0·3	13·1	—	—

In individual fruits during the time that the amount of starch was decreasing, the correlation between starch (g. per cell) and respiration rate (mg. CO_2 per cell per hour) was significant when averaged over picks 8 and 9 (+0.87 on 5 degrees of freedom, $P =$ approx. 1 per cent.) but was not significant when averaged over picks 10, 11, and 12. However, as will be discussed later, the climacteric rise in respiration follows a few weeks after the beginning of starch breakdown, which must be connected in some way with the characteristic metabolic change taking place in the cells at about 190 days from full blossom.

(iii) *Total Organic Acids*.—Total organic acids per cell, calculated as malic acid, increased from 0.3×10^{-8} g. at full blossom to 3.2×10^{-8} g. at 102 days from full blossom. These figures were very similar in the 1949-50 and 1950-51 seasons. After 102 days in tree 2 of the 1949-50 season the acids increased less rapidly to 4.4×10^{-8} g. at 190 days and remained at this level, with slight fluctuations till the end of the season (Table 6, Fig. 5). The decrease in dry weight at 210 days in tree 3 was due partly to decrease in acids; later in the season the concentration of acids increased again, which indicated a synthesis in the cells. The increase in acids in the 1950-51 season from 3.3×10^{-8} g. to 4.2×10^{-8} g. between 190 days and the end of the season (244 days) may be real but the data were not sufficient to test the significance properly. From

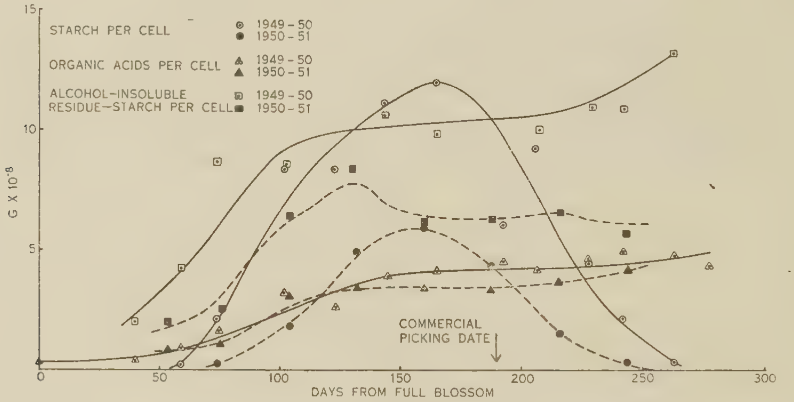

Fig. 5.—Changes in starch per cell, total organic acids per cell, and cell wall material with time.

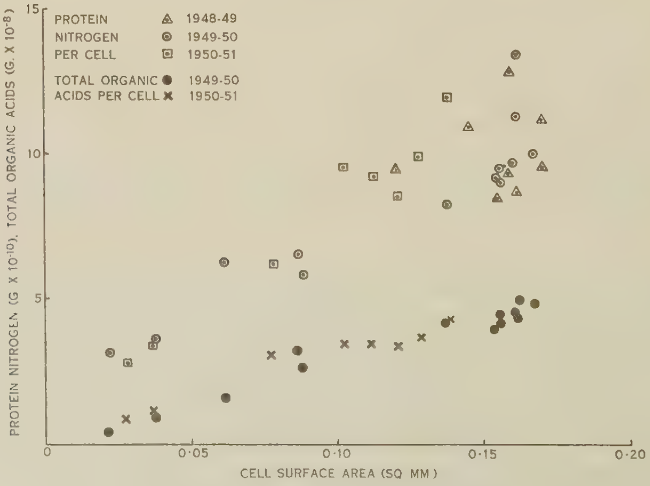

Fig. 6.—Relationship between protein nitrogen per cell, total organic acids per cell, and cell surface area.

comparison with both trees of the previous season it seems that until about the beginning of the climacteric rise in respiration the acids increase in the cells to a certain level, which is maintained during the climacteric and post-climacteric periods. When plotted against mean cell surface, the total acid per cell showed a linear relationship (Fig. 6).

TABLE 6

CHANGES IN TOTAL, PROTEIN, AND SOLUBLE NITROGEN AND TOTAL ORGANIC ACIDS PER CELL WITH TIME

Pick	Days from Full Blossom	Fruit Weight (g.)	Total Nitrogen per Cell ($g. \times 10^{-10}$)	Protein Nitrogen per Cell ($g. \times 10^{-10}$)	Soluble Nitrogen per Cell ($g. \times 10^{-10}$)	Total Organic Acids per Cell ($g. \times 10^{-8}$)
1948-49						
1	129	116·29	19·4	9·4	10·0	
2	155	153·67	23·0	10·8	12·2	
3	169	179·13	19·8	8·4	11·4	
4	183	197·13	—	8·6	—	
5	198	195·75	18·3	9·3	9·0	
6	211	202·91	18·7	9·5	9·2	
7	225	217·01	25·2	11·1	14·1	
8	239	228·08	36·3	12·8	23·5	
1949-50						
1	0	0·106	2·1	1·2	0·9	0·3
2	40	7·75	5·7	3·1	2·6	0·4
3	59	24·73	8·2	3·6	4·6	0·9
4	74	42·97	12·3	6·2	6·1	1·6
5	102	81·85	14·2	6·5	7·7	3·2
6	123	123·84	11·7	5·8	5·9	2·6
7	144	149·99	14·8	9·1	5·7	3·9
8	165	192·84	16·1	8·2	7·9	4·1
9	193	250·01	18·6	9·0	9·7	4·4
10	207	246·38	19·8	9·4	10·4	4·1
11	228	245·88	24·2	9·6	14·5	4·5
12	242	296·51	25·9	11·2	14·7	4·9
13	263	267·25	33·1	9·9	23·2	4·8
14	277	257·60	32·3	13·4	18·9	4·3
1950-51						
1	54	13·95	4·9	2·8	2·1	0·8
2	76	27·79	6·6	3·4	3·2	1·1
3	104	66·05	16·9	6·1	10·8	3·0
4	132	98·97	20·6	9·5	11·2	3·4
5	160	132·12	19·0	9·2	9·8	3·4
6	188	170·28	18·3	8·5	9·8	3·3
7	216	193·01	25·3	9·9	15·4	3·6
8	244	182·78	36·4	11·9	24·4	4·2

(iv) *Alcohol-insoluble Residue Minus Starch.*—This fraction represented an approximate measure of the cell wall material. It increased throughout the season but less rapidly towards the end when the cells were not changing very much in volume (Table 5, Fig. 5). It showed an approximately linear relationship with cell surface, as in the earlier work, indicating that as the cell wall increased in area it remained approximately constant in thickness.

(v) *Total Nitrogen.*—The results of total nitrogen estimations (g. per cell) for the three successive seasons are plotted against time in Figure 7. The trends in each season were similar, showing a steady increase in nitrogen in the cells during the early growing period. Late in the season, another rapid increase in nitrogen made the final concentration at 277 days double that at 190 days from full blossom. In two seasons there was an apparent decrease in total nitrogen for about 60 days prior to commercial picking. Tree 3 in 1949-50 behaved similarly because the intake of nitrogen stopped at 170 days and the amount per cell decreased for 6 weeks, after which there was another rapid intake; the decrease of total nitrogen corresponded with the loss of dry weight mentioned earlier.

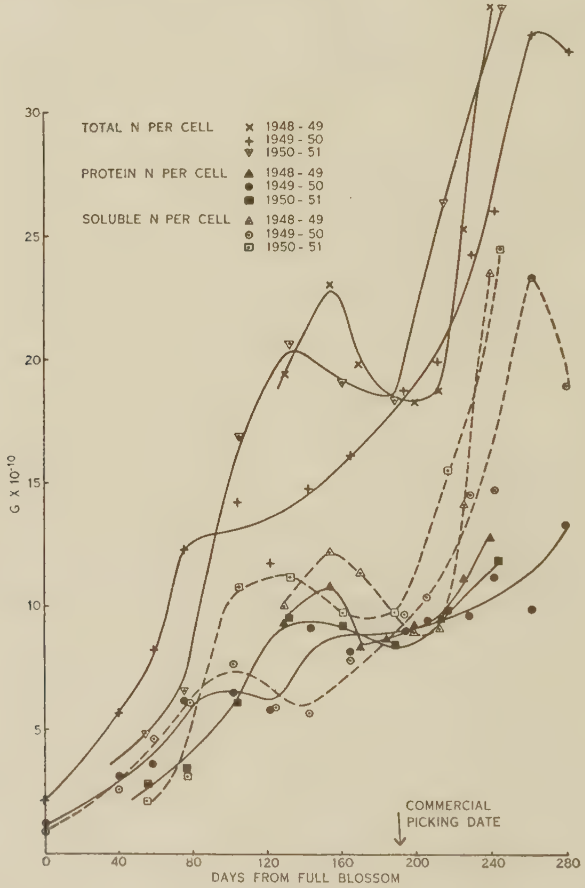

Fig. 7.—Changes in total nitrogen per cell, protein nitrogen per cell, and soluble nitrogen per cell with time.

(vi) *Protein Nitrogen.*—Protein nitrogen (g. per cell) made up approximately 50 per cent. of the total nitrogen per cell during enlargement (Table 6, Fig. 7). Amount of protein nitrogen per cell followed similar trends in all seasons. After about 120 days from full blossom, no further increase in protein content in the cells was observed for about 90 days. From 210 to 280 days from full blossom, further synthesis of protein took place. In tree 3 in 1949-50, the fall in total nitrogen content of the cells was followed by a reduction in protein nitrogen in the picks at 193 and 207 days from full blossom.

(vii) *Soluble Nitrogen.*—The soluble nitrogen content changed in much the same way as the protein nitrogen up to 190 days from full blossom (Fig. 7). For about 50 days before the climacteric rise in respiration, the amount of soluble nitrogen did not increase in the cells. However, after the climacteric rise began, there was a rapid import of soluble nitrogen by the cells and this was followed by further protein synthesis. The rapid increase in soluble nitrogen during the last 70 days was observed in both trees in all seasons.

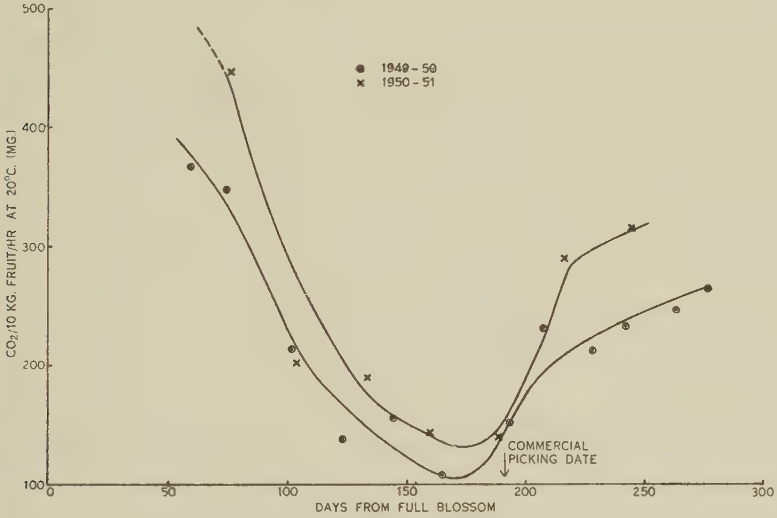

Fig. 8.—Changes in respiration rate per unit weight of fruit with time.

(viii) *Respiration Rate.*—Respiration rate was determined from the mean of seven individual fruits measured separately in the 1949-50 season and from two bulk samples, each of 10 fruits, in the 1950-51 season. The results were calculated per unit weight, per fruit, and per cell. From a comparison of the percentage difference in results from individual fruit of the same pick, the variation when the results were expressed per cell was very much smaller than either per fruit or per unit weight. For this reason, calculation of respiration rate per cell was found to be a very satisfactory means of comparing respiration rates between fruits of different sizes and between fruits of different picks.

In the 1949-50 season, respiration per unit weight of fruit, which is the usual way of expressing the rate, decreased rapidly from about 3300 mg.

CO_2/10 kg./hr. at full blossom to a minimum of 110 mg./10 kg./hr. at 160 days from full blossom. At about the date of commercial maturity of the fruit (190 days) a climacteric rise began and the respiration continued to rise slowly till the end of the season at 277 days (Fig. 8). In Figure 9 all measurements of respiration rate during the 70 hr. after each sampling are plotted to indicate the variation in rate during that time. The line joining each pick has been drawn through the mean. The curves would have been essentially the same in form if drawn through the highest or lowest values in each pick. The same type of drift over the 70 hr. was observed in individual fruits within a pick. The amount of variation in the readings from individual fruits seemed to increase with the age of the fruit and was most marked about the time of the climacteric rise.

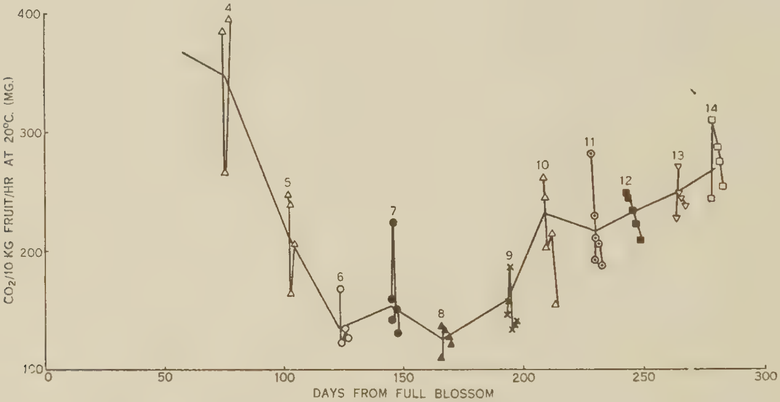

Fig. 9.—Changes in respiration rate per unit weight with time, showing the drift in the readings from which the mean value of each pick was obtained.

Within each pick respiration per cell was approximately proportional to cell volume. Fruits of different weights but similar cell volumes showed almost the same respiration rates per cell but more variable respiration rates per fruit or per unit weight. From Table 7 it is evident that calculation of respiration rate per fruit and per unit weight did not give comparable results between fruits of the same pick or between picks, and that respiration per cell gave the only really satisfactory means of expression.

The climacteric rise is seen clearly when expressed as respiration per cell. These results are given in Table 8 and plotted against time in Figure 10. In 1949-50 respiration rate per cell rose from 1.3×10^{-8} g. CO_2 per cell per hour at full blossom to 8.7×10^{-8} g. CO_2 per cell per hour at 193 days. Within the next 14 days it rose to 14.1×10^{-8} g., then increased slowly to 17.1×10^{-8} g. CO_2 per cell per hour at 277 days. In the later season respiration rate per cell followed the same pattern with the climacteric rise occurring at the same time as in both earlier seasons and at the same respiration rate per cell. It was not possible from the results to tell whether the increase in rate took place over a period of a few days or of 2-3 weeks, since no intermediate samples were taken.

IV. DISCUSSION

(*a*) *Cell Size*

The observations on mean cell size over three successive seasons confirm the principal conclusions of Bain and Robertson (1951), but with fruit kept on the tree till the end of the season, the increase in cell volume is progressively diminished after about 190 days from full blossom. This was not observed by Bain and Robertson, whose observations ceased at 200 days. The fruits of one season (1950-51) were smaller and the cell volumes less than those of the other two seasons.

TABLE 7

COMPARISON OF RESPIRATION EXPRESSED AS RATE PER CELL, RATE PER FRUIT, AND RATE PER UNIT WEIGHT

Pick No.	Fruit Weight (g.)	Cell Volume (cu.mm.)	CO_2/ Cell/Hr. at 20°C. (mg. $\times 10^{-7}$)	CO_2/ Fruit/ Hr. at 20°C. (mg.)	CO_2/ 10 Kg./ Hr. at 20°C. (mg.)	Variability Within Each Pair as a Percentage of the Mean when Expressed as:		
						Respiration per Cell	Respiration per Fruit	Respiration per Unit Weight
7	153·1	0·0041	0·81	2·50	147·86	1·23	4·50	17·60
	138·6	0·0040	0·82	2·39	176·40			
9	292·5	0·0058	1·00	4·26	150·14	1·95	45·11	2·22
	172·4	0·0054	1·02	2·69	153·51			
9	192·0	0·0062	1·09	2·87	159·67	0·92	18·25	5·78
	153·7	0·0058	1·08	2·40	150·70			
9	255·6	0·0067	1·06	3·43	134·12	0	14·59	2·17
	302·9	0·0067	1·06	3·97	131·24			
10	266·0	0·0058	1·53	5·92	222·44	0·65	19·46	9·66
	198·7	0·0052	1·54	4·87	245·03			
10	257·5	0·0065	1·65	5·39	195·15	0	27	7·02
	309·2	0·0059	1·65	7·06	209·34			
11	258·6	0·0062	1·49	5·17	200·03	0·67	11	8·26
	252·2	0·0068	1·48	4·64	184·16			
13	279·3	0·0060	1·63	6·49	232·29	0	6·85	10·35
	235·4	0·0055	1·63	6·06	257·64			
13	279·3	0·0060	1·63	6·49	232·29	3·0	7·85	7·50
	252·6	0·0059	1·68	6·00	250·38			

An increase in calculated cell number during the period of cell enlargement was observed in the three seasons discussed in this paper but not by Bain and Robertson (1951). We do not know whether this increase was real, and due to the persistence of a small amount of cell division, or was apparent, and due to the method of calculating cell numbers. The variation in cell number between picks was wide and erratic and will not be discussed further until more information has been obtained.

TABLE 8

CHANGE IN RESPIRATION RATE PER CELL WITH FRUIT WEIGHT AND TIME IN TREE 2

Days from Full Blossom	1948-49		1949-50		1950-51	
	Fruit Weight (g.)	CO_2/Cell/ Hr. at 20° C. (mg. $\times 10^{-8}$)	Fruit Weight (g.)	CO_2/Cell/ Hr. at 20° C. (mg. $\times 10^{-8}$)	Fruit Weight (g.)	CO_2/Cell/ Hr. at 20° C. (mg. $\times 10^{-8}$)
0	—	—	0·106	1·30	—	—
40	—	—	7·75	2·86	—	—
54	—	—	—	—	13·95	5·73
59	—	—	24·73	2·87	—	—
74	—	—	42·97	5·58	—	—
76	—	—	—	—	27·79	3·05
102	—	—	81·85	5·64	—	—
104	—	—	—	—	66·05	4·49
123	—	—	123·84	4·09	—	—
129	116·29	5·18	—	—	—	—
132	—	—	—	—	98·97	6·35
144	—	—	149·99	7·04	—	—
155	153·67	6·98	—	—	—	—
160	—	—	—	—	132·12	4·64
165	—	—	192·84	6·22	—	—
169	179·13	6·40	—	—	—	—
183	197·13	7·37	—	—	—	—
188	—	—	—	—	170·28	5·10
193	—	—	250·01	8·68	—	—
198	195·75	8·68	—	—	—	—
207	—	—	246·38	14·11	—	—
211	202·91	8·96	—	—	—	—
216	—	—	—	—	193·01	15·61
225	217·01	11·84	—	—	—	—
228	—	—	245·88	14·23	—	—
239	222·08	11·38	—	—	—	—
242	—	—	296·51	13·29	—	—
244	—	—	—	—	182·78	16·22
263	—	—	267·25	15·81	—	—
277	—	—	257·60	17·05	—	—

(*b*) *Nitrogen Compounds*

The changes in amount of the nitrogen compounds during the three seasons confirm the observations recorded by Robertson and Turner (1951). Total and protein nitrogen per cell increase with increasing cell volume in the preclimacteric period. Within a pick the fruits with the larger cell volume had more protein nitrogen per cell; for example in pick 8, the fruit with the greatest cell volume, 0.0058 cu. mm., had the highest protein nitrogen, 0.101×10^{-8} g. per cell. In the same pick the fruit with the smallest cell volume, 0.0043 cu. mm., had the lowest protein nitrogen per cell, 0.071×10^{-8} g. per cell. Similarly the fruits with the lower cell numbers, which were mostly those with the larger cells, had the highest protein nitrogen per cell.

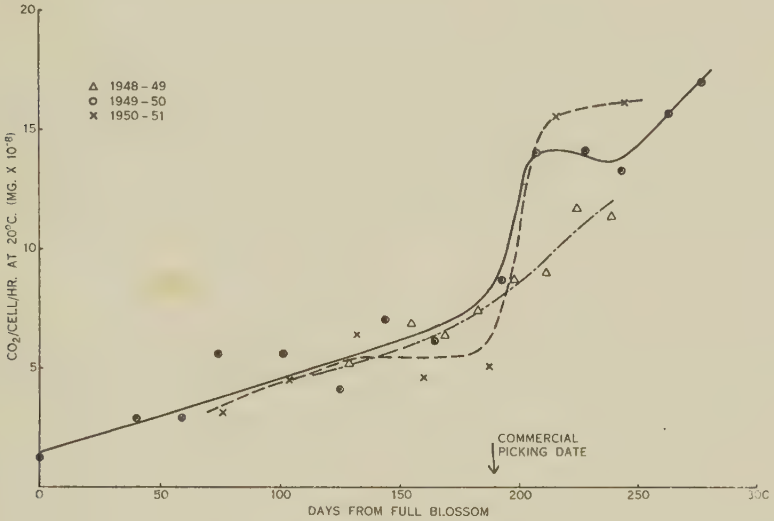

Fig. 10.—Changes in respiration rate per cell with time.

If protein nitrogen can be taken as a measure of cytoplasmic content, and if, as suggested by Robertson and Turner (1951), the cytoplasm increases with increase in cell surface but remains approximately constant in thickness, a linear relationship between protein nitrogen per cell and cell surface would be expected. This linear relation was obtained in both seasons, which confirms this interpretation. The results from tree 2 are shown in Figure 6. The results from later picks of tree 3 in 1949-50 did not show the linear relationship but this was explained by the marked drop in protein nitrogen at the same time as the decrease in dry weight; after this temporary setback, protein nitrogen increased. The last sample for both trees was high in protein nitrogen, suggesting increased rate of protein synthesis at the end of the life of the fruit on the tree. Protein synthesis was relatively more rapid than increase in dry weight in the last two samples. If, then, the protein nitrogen level is a true measure of cytoplasmic protein, the cytoplasm forms a layer of constant thickness inside the cell wall during cell expansion, but protein apparently may continue to increase later when increase in cell surface is only small.

Mean protein nitrogen and mean soluble nitrogen were correlated during growth up to 190 days with a correlation coefficient of + 0.844 (with 7 degrees of freedom). At commercial maturity, protein and soluble nitrogen contents were correlated, thus being consistent with the results of Robertson and Turner (1951). After this time, however, the soluble nitrogen increased rapidly relative to the protein nitrogen. This was not recorded by Hulme (1951) whose observations ceased at 160 days, or by Robertson and Turner (1951) whose observations were not continued beyond 205 days. This increase in soluble nitrogen may be the result of protein degradation in the senescent leaves supplying the fruits. The composition of the soluble nitrogen fraction is now being studied.

In all three seasons, there is evidence that both protein and soluble nitrogen, which have been increasing up to 120 days, then remain stationary until 190 days when the soluble nitrogen again increases rapidly. After 190 days, the protein nitrogen also increases, but only slowly. The check in the increase of protein and soluble nitrogen after about 120 days varied in the different seasons. In 1950-51, the increase in protein appeared to lag behind the increase in soluble nitrogen. The lack of net protein synthesis at a time when soluble nitrogen content is high shows that some factor other than nitrogen supply controls protein synthesis, and possibly cell enlargement, at this stage.

(*c*) *Organic Acids*

The total organic acids per cell increased, as observed by Robertson and Turner, up to 190 days from full blossom, but did not increase markedly during the remainder of the season when the nitrogen compounds increased so rapidly and when the respiration rate increased in the climacteric. At this period, however, an increase in acids in the fruit of one tree showed a capacity for synthesis. Mean total organic acids per cell were correlated with mean protein nitrogen per cell over the whole period with a coefficient of $+0.93$ (with 12 degrees of freedom). This confirms the high correlation found by Robertson and Turner in samples of fruit up to commercial maturity. In all living material investigated the acids form the connecting link between the respiration mechanism and protein synthesis. Turner (1949) found evidence of a tricarboxylic acid cycle in mature Granny Smith fruits. It is possible, as suggested by Robertson and Turner (1951), that the increase in acid content with increase in protein content in developing fruit can be explained by a steady-state relation between acids and nitrogen compounds. Perhaps, towards the end of the life on the tree when the respiration has increased, the steady-state organic acid content does not change, an increased rate of formation being offset by an increased rate of utilization, due both to protein synthesis and to use as additional substrates in respiration. Homogenates of apple tissue in the present season (1951-52) show that there is an increase in enzymes capable of oxidizing organic acids at the time of the onset of the climacteric. Kidd *et al.* (1951) found that the rate of metabolism of malic acid in Worcester, Pearmain, and Bramley's Seedling apples was unaffected by the onset of the climacteric rise, which agrees with our observation that the total organic acid content does not change at the time of the climacteric rise.

A linear relationship between cell surface and total organic acids was obtained in this work (Fig. 6) and agrees with the observations of both Kidd *et al.* (1951) and Robertson and Turner (1951). The latter authors interpret the high correlation of protein nitrogen with total organic acids and of total acids with cell surface to indicate a steady-state relationship between protein nitrogen, organic acids, and respiration. Kidd *et al.*, however, suggest that the organic acids are not related to protein synthesis but are formed as a side reaction of the metabolism of cell wall formation. Evidence from our data is insufficient to decide between the two hypotheses but would favour the Robertson and Turner interpretation as it appears that the total acids continue to be formed in the late picks after cell wall formation has ceased.

(*d*) *Respiration and the Climacteric Rise*

Robertson and Turner (1951) found some evidence of a climacteric rise in respiration at the end of their experiment. In all three seasons discussed in this paper, a climacteric rise in respiration took place at about 190 days from full blossom, at the time of commercial maturity. A number of metabolic changes occur in the cells about the time of the climacteric rise and provide further information as to the possible cause.

Prior to the onset of the climacteric rise in respiration, protein nitrogen per cell was related to respiration rate per cell. In individual fruits those with larger cells had a high protein nitrogen per cell and a high respiration rate per cell. Hulme (1951) has shown that the ratio of respiration rate to protein nitrogen content is reasonably constant during development from a period soon after cell division until the onset of the climacteric. Our data over the two complete seasons agree approximately with this, but in 1949-50 season this ratio for the mean respiration rates and protein nitrogen contents in the different picks decreased from 0.9 to 0.76 (not significant at $P=0.05$) and in the 1950-51 season from 0.9 to 0.6 (just significant at $P=0.05$), before the onset of the climacteric rise. The 12 observations, over the 2 years, taken together, are homogeneous and the decrease is significant (at $P=0.01$).

In contrast, at the climacteric, the increase in respiration rate was not accompanied by a corresponding increase in protein nitrogen. A number of workers (e.g. Gregory and Sen 1937) have found a relation between respiration rate and protein nitrogen during growth but the mechanism of control has not yet been worked out. The difference between preclimacteric and climacteric cells in our work suggests that the factors controlling respiration rate and protein synthesis at the two stages are different. The protein nitrogen level was the same in all seasons at the time of the climacteric rise and a small increase in protein took place after the rise. Since both soluble nitrogen and respiration rate are considerably increased after the climacteric rise, net protein formation must be limited by some other factor. Possibly the transfer of energy even from the increased respiration is sufficient only for the resynthesis of the unstable protein in the cells by that time; thus at higher nitrogen contents the turnover of nitrogen would be increased without the net protein content increasing in proportion.

Other observations on the climacteric rise confirm those of Robertson and Turner (1951). The increasing protein turnover in synthesis and degradation may result in the increase in respiration rate, because, as Robertson and Turner suggest, the more rapid removal of phosphates from the phosphate carrier systems, thus freeing phosphate acceptors, would probably increase respiration rate. The increase in respiration rate is being studied further, both in cut tissue and in homogenates from apples of different maturities. Some evidence has been obtained which supports the hypothesis and will be published soon.

V. ACKNOWLEDGMENTS

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CHEMICAL STUDIES ON *EUCALYPTUS REGNANS* F. MUELL.

By C. M. Stewart*, G. L. Amos*, and L. Josephine Harvey*

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Summary

Samples of the outer phloem, inner phloem, cambial zone, developing sapwood, sapwood, and heartwood of young *Eucalyptus regnans* F. Muell. trees have been examined for extraneous materials, lignin, and carbohydrate constituents.

The hexose carbohydrate increases from less than 12 per cent. in the cambial zone to less than 46 per cent. in the outer phloem and to less than 58 per cent. in the xylem. Hexose carbohydrate deposition is essentially completed in the developing sapwood.

The apparent Klason lignin increases from 12 per cent. in the developing sapwood to 25 per cent. in both the outer phloem and the heartwood; however, the calculated "true" lignin rises from less than 1 per cent. in the cambial zone to about 10 per cent. in the phloem and to about 25 per cent. in the mature xylem. The calculated values indicate that lignification commences in the developing sapwood and is completed in the sapwood.

The ratio of pentosan/uronic acid anhydride is 2/1 in the phloem and 6/1 in the xylem, being about 1/1 in the cambial zone. Very little of the uronic acid anhydride is associated with pectinous material.

The concentration of sugars in the fluids of the inner phloem, cambial zone, and developing sapwood is of the order of 1 per cent., while that in the sapwood and heartwood is very low: this suggests that rapid cell growth is restricted to the cambial-developing sapwood region.

In the sapwood and heartwood there is, respectively, sufficient and insufficient water to fill all "free" spaces. The aqueous phase in the cambial zone contains at least 1 per cent. of inorganic salts. All tissues are slightly acidic, the strongest acids occurring in the outer phloem.

The extraction of the zonal samples with 80 per cent. ethanol causes very little hydrolytic degradation and the undifferentiated and partly differentiated elements are rich in ethanol-soluble materials.

Tannins are present in minimal amounts in the cambial region and their concentration in the "sap" fluids increases on either side of the cambium.

Proteins are plentiful in the cambial and adjacent tissues. The relatively high protein, sugar, and soluble mineral matter contents of the outer phloem are consistent with the observation that the "bark" of the samples examined is composed largely of living cells.

The effect of the method of drying the samples on subsequent analytical data indicates that it is inadvisable to employ material oven-dried at 105°C. or thereabouts.

I. Introduction

Few workers have studied the chemical changes that occur in the cell wall material during differentiation and maturation of elements derived from the cambium in woody species. Allsopp and Misra (1940) have analysed the cam-

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bium, new wood, and sapwood of *Fraxinus elatior* L., *Ulmus sativa* Mill., and *Pinus silvestris* L., while Anderson and Pigman (1947) have investigated the outer bark, inner bark, cambial zone, young sapwood, sapwood, and heartwood of *Picea mariana* (Mill.) B.S.P. In the present work, the above-mentioned studies have been extended to *Eucalyptus regnans* F. Muell.

The mode of synthesis of sugars and proteins in leaves has been the subject of much research work and speculation. Turner (1951), in a recent review, has summarized the modern conceptions of photosynthesis. It is generally agreed that sugars and other materials synthesized in the leaves are transported, through the "sap" stream in the phloem, to the cambial region where the nutrient materials are utilized during growth of the wood and bark elements.

Schwalbe and Neumann (1930) found reducing sugars, uronic acids, oxalic, succinic, and citric acids, guaiacol, and vanillin in the saps of *Picea abies* (L.) Karst., *Pinus silvestris* L., and *Fagus silvatica* L. Wiesner (1928) stated that nearly all hardwoods and softwoods have a high glucose content in the spring. Schwalbe and Ender (1931) found that 85 per cent. of the solid material from an extract of *Fagus silvatica* is glucose.

Klason (1929) and Wislicenus and Hempel (1933) found high concentrations of sucrose in the saps of *Picea abies* and young *Quercus* spp. respectively. Anderson and Pigman (1947) isolated 33 per cent. of crystalline sucrose (on a dry basis) from the cambial zone of *Picea mariana,* and found considerable amounts in the inner bark. Allsopp and Misra (1940) have shown that, for *Pinus silvestris, Fraxinus elatior,* and *Ulmus sativa,* "the free reducing substances formed only a comparatively small proportion of the alcohol extract of the cambium, which had, however, a much higher sucrose content." Kurth (1944) has shown that many woods contain oils, fats, waxes, acids, phytosterols, pigments, tannins, phlobaphenes, water-soluble polysaccharides, cyclohexanols, saponins, and mineral constituents.

Because of the preliminary nature of the present work, the above-mentioned extraneous substances have not been studied in detail; however, the wood substance from the various zones of *E. regnans* has been examined in greater detail. The conclusions drawn must be regarded as tentative because analyses, of portions of one or a few trees, concerning the development of wood tissues are necessarily doubtful for general application to the species as a whole (Stewart *et al.* 1951).

II. SAMPLE PREPARATION

The samples were taken, shortly after the resurgence of cambial activity, from six 8-year-old trees of *E. regnans* grown at Toolangi, Victoria. The freshly cut logs were transported to the laboratory and divided into six zones, viz., outer phloem*, inner phloem*, cambial zone, developing sapwood, sapwood, and heartwood. The logs peeled readily by rupture at the cambial layer, and the phloem was divisible into two parts, an inner white portion and an outer green and brown portion. Separation was effected by hand and the strips of inner and outer phloem were cut into small pieces—approximately 1½ in. long by ½ in. wide.

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* The whole of the bark sampled from this species consisted of living cells, and therefore must be regarded as phloem (Chattaway, unpublished data).

A "Perspex" scraper was made with the edges concavely shaped at various radii to accommodate logs of different sizes. By running the scraper down the log and applying some pressure, thin strips of partly differentiated material, five or six cells thick, were removed from the surface; these strippings have been called the "cambial zone." To minimize the loss of a fine spray, which shoots out from the log, the scraper was kept inclined at an acute angle to the surface of the log. During stripping, the log was held vertically with the lower end in a copper dish in which the strippings and the exuded liquid were collected. Before stripping with the scraper, a small amount of material from the cambial zone was removed with a spoon for the determination of volatile substances by method (*a*)(i) (below); these samples were taken at several points around the circumference and at various levels, the scrapings being combined for the determination of volatile substances. About 15 6-ft. logs from the six trees were required to provide sufficient cambial material for chemical study.

An attempt to remove material from the inner phloem in the same way was not successful, indicating that peeling of the phloem does not remove any undifferentiated tissue, at least during the early spring. This fact, and the evidence from microscopic examination, justifies the term "cambial zone" for the superficial strippings from the peeled log.

Following the removal of the partly differentiated material, shavings about 1/32 in. thick were taken with a spokeshave from the new surface of the log. The shavings (designated "developing sapwood") were cut into small pieces (1½ in. long by ½ in. wide).

Immediately after removal, all the above samples were subdivided. One portion was rapidly weighed and plunged into sufficient boiling ethanol to produce a solution containing about 80 per cent. ethanol after taking account of the water contained in the sample; all enzyme activity was immediately inhibited thereby. The rest of the cambial zone and further portions of the other three outer zones were dried at 105°C. in an air oven until constant in weight. The remainder of the outer phloem, inner phloem, and developing sapwood was air-dried at room temperature.

Many tests have shown that, in *E. regnans* of this age and from this locality, the outer three growth rings are sapwood and the remainder are heartwood. Hence sapwood samples were taken from the outer two growth rings and heartwood samples from the innermost three rings. Representative disks were removed from each log and the sapwood and heartwood (excluding pith) samples were cut with a band saw. Samples for the determination of volatile substances were taken and the remainder dried at about 40°C.; the samples were then comminuted in a Wiley mill to pass a 60-mesh sieve. All other air-dried and oven-dried samples were similarly milled.

All the samples that had been plunged into boiling ethanol and portions of the air-dried sapwood and heartwood were extracted three times with 80 per cent. ethanol under 8-hr. reflux and then dried in a vacuum oven at about

45°C. After ethanol extraction, the solid residues from the four outer zones were comminuted to pass a 60-mesh sieve. The ethanol extracts of the various zones were measured and bottled for subsequent examination.

III. EXPERIMENTAL METHODS

(a) *Volatile Substances (including water)*

The volatile substances content was determined in three ways:

(i) Small representative samples from each zone were taken in duplicate immediately upon removal from the log, dried for 16 hr. in the vacuum oven at about 45°C., and finally heated to constant weight in an air oven at 105°C.

(ii) Large representative samples from each of the four outer zones were dried in an air oven at 105°C. until constant in weight.

(iii) The sum of the ethanol-soluble material and the vacuum-dried ethanol-extracted residue was subtracted from the total fresh weight of the sample extracted.

(b) *Distribution of Volatile Substances across the Diameter of the Tree*

In a separate experiment, trees were felled at monthly intervals and disks immediately removed from breast height. Two sectors were taken from the north and south sides of each disk and the sectors were split into early wood and late wood for each growth ring. The samples were immediately weighed and then dried for 16 hr. in a vacuum oven at about 45°C. and finally to constant weight in an air oven at 105°C. The average results for November, December, and January are diagrammatically presented in Figure 1. Subsequent results for June were not significantly different from the results in Figure 1, showing that the distribution of volatile substances does not exhibit appreciable seasonal variation in this species.

(c) *Mineral Constituents*

The distribution of mineral constituents was determined by micro-incineration at 550°-600°C. of thin transverse sections on glass slides (Plate 1, Figs. 1, 2, and 3), and their nature by spectrographic analysis. The mineral constituents were determined quantitatively on the non-volatile ethanol-soluble materials, on the ethanol-extracted materials, and on the unextracted samples by ignition at 575° ± 25°C.

For the chloride determinations, 500 ml. of each of the 80 per cent. ethanol extracts were evaporated to dryness. The residues were ignited at 575° ± 25°C. and the residual ashes extracted with water. Aliquots of the filtrates were titrated against 0.05N $AgNO_3$ using 0.1 per cent. dichlorofluorescein solution as indicator (Mohr method). The Volhard method was applied to aliquots from the cambial and immediately adjacent zones: the titres were in good agreement with those obtained using the Mohr method.

(d) *Total Non-volatile Solids in Ethanol Extracts*

Aliquots from the ethanol extracts were evaporated on a steam bath and finally dried for 2 hr. in the air oven at 105°C.

(*e*) *Acids in Ethanol Extracts*

(i) *Volatile Free Acids.*—Aliquots of the ethanol extracts were distilled at 100°C. and the distillate titrated against 0.1N NaOH with phenolphthalein as indicator.

(ii) *Volatile Esters.*—Aliquots of the ethanol extracts were distilled at 100°C. and the distillate was then mixed with 50 ml. of 0.1N NaOH and refluxed for 16 hr. The mixture was then back-titrated with 0.1N HCl, using phenolphthalein as indicator. The titre was corrected for volatile free acids.

(iii) *Non-volatile Free Acids.*—Aliquots of the ethanol extracts were titrated potentiometrically against 0.1N NaOH to pH 9.5. The titres were corrected for volatile free acids.

(*f*) *Hot-water Solubles*

About 3 g. of each sample was heated at about 95°C. for 1 hr. with 300 ml. of water. The solid material was then rapidly filtered on an alundum crucible (RA 98 porosity), washed, dried in a vacuum oven at 45°C., and weighed. With the cambial material, where the suction filtration was too slow, the solid residue was separated by centrifuging.

(*g*) *Tannins*

The 80 per cent. ethanol extracts and the hot-water extracts from the oven-dried and 80 per cent. ethanol-extracted samples were examined. With the ethanol extracts the ethanol was removed by distillation under vacuum at 45°C. and replaced by water. Suitable volumes of the extract were analysed by the Löwenthal method (Clowes and Coleman 1938). The tannin value of the potassium permanganate solution was estimated on the assumption that 1 g. of gallic acid is equivalent to 1.7 g. of *E. regnans* tannin.

(*h*) *Sugars in Ethanol Extracts*

The ethanol was removed by distillation under vacuum at 45°C. and replaced by water. The aqueous solution of sugars was then clarified by treatment with excess neutral lead acetate, followed by treatment with anhydrous sodium carbonate.

(i) *Reducing Substances Calculated as Glucose.*—A 50 ml. aliquot of the neutral clarified solution was pipetted into a 400 ml. beaker containing 25 ml. each of Fehling's solutions A and B.

The precipitation of cuprous oxide was carried out according to method 34.39 of the A.O.A.C. "Methods of Analysis" (1945). The washed precipitated oxide was dissolved in ferric ammonium sulphate solution (100 g. ferric alum and 140 ml. conc. H_2SO_4 per litre), and the liberated ferrous ions were titrated at about 50°C. against 0.05N ceric sulphate solution, using xylene cyanol FF as indicator. A blank was made in a similar manner. The quantity of cuprous oxide formed was converted to the equivalent amount of sugar by the use of the conversion table of Munson and Walker (1906).

(ii) *Invertible Substances Calculated as Sucrose.*—Acid and invertase inversions were used. For the *acid inversion*, a 50 ml. aliquot of the clarified solution was inverted as described under method 34.24 (*b*) of the A.O.A.C. "Methods of Analysis" (1945). After the inversion the reducing substances were determined as described above.

For the *invertase inversion*, a 100 ml. aliquot of the clarified solution, and enough acetic acid to render the solution distinctly acid to methyl red indicator, were added to a 250 ml. measuring flask. Invertase solution (12.5 ml.) and water were added to fill the flask almost to the mark. The flask, after standing for 16 hr. at about 20°C., was filled to the 250 ml. mark and the reducing substances were determined as described above.

(*i*) *Nitrogenous Materials*

The comminuted zonal samples were dried at 45°C. for 16 hr. in a vacuum oven. The analyses were performed according to methods 2.22, 2.23, 2.24, 2.29, and 2.27(2) of the A.O.A.C. "Methods of Analysis" (1945).

(*j*) *Klason Lignin*

(i) *On Solid Material.*—The Klason lignin was determined by the method of Ritter, Seborg, and Mitchell (1932) with minor modifications developed by Cohen and Dadswell (1931), Cohen (1934, 1936), and Meade (unpublished data).

(ii) *On* 80 *per cent. Ethanol Extracts.*—Method (i) was used after first evaporating to dryness 500 ml. of each 80 per cent. ethanol extract.

(*k*) *Methoxyl*

These determinations were made according to the method of Vieböck and Schwappach (1930) with minor modifications (Clark 1932).

(*l*) *Alkaline Nitrobenzene Oxidations*

The method of Creighton, McCarthy, and Hibbert (1941) was used.

(*m*) *Carbohydrate Constituents*

(i) *Galactose.*—Samples of various zonal materials were treated according to the method of Schorger (1926). As the results obtained were rather variable, not much reliance should be placed upon them.

(ii) *Uronic Acid Anhydride.*—Two methods of estimation were used, viz.: firstly, the method of Dickson, Otterson, and Link (1930), as modified by Campbell, Hirst, and Young (1938); and, secondly, the method of Whistler, Martin, and Harris (1940).

(iii) *Pentosan.*—Pentosan was estimated by a method similar to that described by Dorée (1933), except that thiobarbituric acid was used instead of phloroglucinol as the precipitant (Mackney and Reynolds 1938). The total pentosan values were corrected, for furfural from uronic acid, according to the values quoted by Norris and Resch (1935).

(iv) *Acetyl.*—This determination was made according to Wise (1944). Because hydrochloric acid, as well as ethyl acetate, was liberated from the samples containing chlorides, the amount of chloride present in the neutralized refluxate was determined and the appropriate correction was applied to each acetyl value.

(v) *Holocellulose.*—Samples, comprising 1-2 g. oven-dried, of the various zonal materials, were treated according to Wise, Murphy, and D'Addieco (1946).

IV. Results and Discussion

In this discussion the analytical results will be considered under the headings:

(*a*) Extraneous substances;
(*b*) Nitrogenous substances; and
(*c*) Wood substance.

Most of the substances soluble in 80 per cent. ethanol are extraneous to the wood substance, i.e. the so-called "lignocellulose." They include substances that eventually become incorporated in the wood substance (sugars, lignin precursors), essential materials for vital processes (sugars, proteins, amino acids, mineral constituents), and metabolic by-products (tannins, kino, etc.). Other extraneous substances undoubtedly remain in the samples extracted with 80 per cent. ethanol.

(*a*) *Extraneous Substances*

It may justifiably be assumed that immediate extraction with boiling 80 per cent. ethanol inhibited enzyme activity. The possibility of acids present in the fresh material from each zone causing hydrolysis is contraindicated in that the 80 per cent. ethanolic extracts contain negligible amounts of pentose or pentosan (Tables 1 and 13). Because wood pentosans are readily hydrolysed it may be assumed that hydrolytic degradation has been very small.

In zones where 80 per cent. ethanol solubles exceed 7 per cent. (Table 1), at least 80 per cent. of them have been accounted for as inorganic substances, sugars, etc. Some of the determinations give minimum values (e.g. inorganic constituents by ignition, non-volatile acids as acetic acid), and materials not determined include waxes, fats, kino, tannin, etc. Part of the kino, tannin, and waxy materials are undoubtedly included in the determination for lignin-like substances; therefore the tannin values, from Table 9, have not been included in the summation in Table 1.

Analyses expressed on the basis of the dry 80 per cent. ethanol-extracted material give a more accurate representation of the chemical composition of the wood substance than the more usual method of expressing the results on the basis of the dry unextracted sample, especially in the outer zones.

(i) *Water Distribution.*—The three methods (Table 2) of drying were used to determine the effect of each on the chemical composition of the material. Preliminary drying in the vacuum oven at 45°C. was done because it had been found that immediate drying at 105°C. gave inconsistent results.

The volatile substances lost during drying, particularly from the cambial zone, include some highly odoriferous substances which, although widely associated with living matter, have received very little attention. If the freshly stripped cambial zone material is steam-distilled or oven-dried, the distillate and vapours are intensely odoriferous, but the compounds responsible have not been identified. Such odoriferous substances are included with water in the determined "volatile substances."

It has been decided to accept the data for volatile substances as determined by method (*a*)(iii) for the four outer zones and by method (*a*)(i) for the two inner zones. Alkoxyl determinations on air-dried and on ethanol-extracted vacuum-dried samples have shown that ethanol is not retained by the material after ethanol extraction; therefore no source of error arises by virtue of the extraction.

TABLE 1

SUMMATION OF THE KNOWN MATERIALS SOLUBLE IN 80 PER CENT. ETHANOL PRESENT IN THE VARIOUS ZONES OF YOUNG *E. REGNANS* TREES

All results are expressed on the basis of the dry, fresh sample

Determination	Zones					
	Outer Phloem (%)	Inner Phloem (%)	Cambial Zone (%)	Developing Sapwood (%)	Sapwood (%)	Heart-wood (%)
Inorganic constituents ..	0·9	0·9	16·0	2·4	0·3	0·1
Reducing substances (as glucose)	1·2	0·9	6·3	1·9	Negligible	Negligible
Invertible substances (as sucrose)	0·5	2·4	6·6	1·2	Negligible	Negligible
Non-volatile acids (as acetic acid) ..	2·3	1·4	2·5	0·5	0·1	—
Lignin-like substances (as Klason lignin) ..	4·3	2·0	5·4	1·0	0·7	1·5
CO_2-yielding substances (as uronic acid anhydrides)	—	0·6	0·6	0·2	—	—
"Proteins"	0·1	0·1	0·6	0·6	—	—
Pentosan or pentose ..	Negligible	Negligible	Negligible	Negligible	Negligible	Negligible
Calculated 80% ethanol solubles (i.e. sum of above values)	9·2	8·3	38·0	7·8	1·1	1·6
Observed 80% ethanol solubles	11·5	10·4	44·5	9·6	1·6	2·7

The high percentage of water in the cambial zone is due to the small amount of structural material present (Plate 2, Figs. 1 and 2), and the lower percentage on either side of it is a reflection of increased differentiation.

In Figure 1 the average moisture contents from the outer ring to the centre of the tree are plotted. A sinuous line is also included to illustrate what is possibly the actual moisture gradient across the tree.

When the samples were taken, the early wood of the outer ring (1947-48) was forming, hence the high water content. In the three earlier growth rings the water content was constant at about 66 per cent. in the early wood and about 61 per cent. in the late wood. These three rings were sapwood, and the basic density was determined to be 20.1 lb./cu. ft. in the early wood and 29.7 lb./cu. ft. in the late wood. Taking the value for the density of wood substance to be 1.53 (Stamm and Hanson 1937), the percentages of water required to fill all free space were 70.8 and 59.5 for early wood and late wood respectively. These were sufficiently close to the experimental values to conclude that, in the living tree, all space in the sapwood not occupied by the wood substance was filled with water, and that a full vessel system was maintained throughout the sapwood. The sap tension in the vessels emptied them near a cut, thereby accounting, in part at least, for the difference between the calculated and experimental moisture contents of the early wood. In the heartwood, the moisture content fell, indicating that gas or vapour replaced some of the water in the free space.

Table 2

VOLATILE SUBSTANCES PRESENT IN THE VARIOUS ZONES OF YOUNG *E. REGNANS* TREES

All results are expressed on the basis of the fresh sample

Zone	Volatile Substances Determined by Various Methods		
	Method (i) (%)	Method (ii) (%)	Method (iii) (%)
Outer phloem . .	65·2	64·5	63·7
Inner phloem . .	64·6	63·3	62·2
Cambial zone . .	95·0	95·0	94·2
Developing sapwood	72·0	76·6	74·9
Sapwood	62·7	—	—
Heartwood . .	55·6	—	—

(ii) *Mineral Constituents.*—The percentage of inorganic salts in the dried cambial zone is much higher in the hardwoods than in the softwoods for which data are available (Table 3), and is highest in *Eucalyptus regnans.* This is due to the smaller amount of structural material in the cambial region in the hardwoods, and not to a higher concentration of inorganic substances in the sap. In Table 3, the maximum concentrations of inorganic materials in the sap, calculated by assuming all inorganic constituents to be soluble, are given in parentheses. These do not show the real position, because some of the inorganic materials are known to be fixed, but for comparative purposes they show a much smaller difference between hardwoods and softwoods than comparison of total percentages of inorganic salts in the dried material. Hence it must be concluded that the hardwoods contain less structural material in the cambial zone.

For *Eucalyptus regnans,* closer approximations to the concentrations of inorganic materials in the sap of the various zones are obtained from the 80 per cent. ethanol-soluble inorganic salt contents (Table 6) and the water contents. These calculations give the following lower limits for concentrations of inorganic matter in the saps:

Outer phloem	0.51 per cent.
Inner phloem	0.52 per cent.
Cambial zone	0.98 per cent.
Developing sapwood	0.82 per cent.
Sapwood	0.16 per cent.
Heartwood	0.05 per cent.

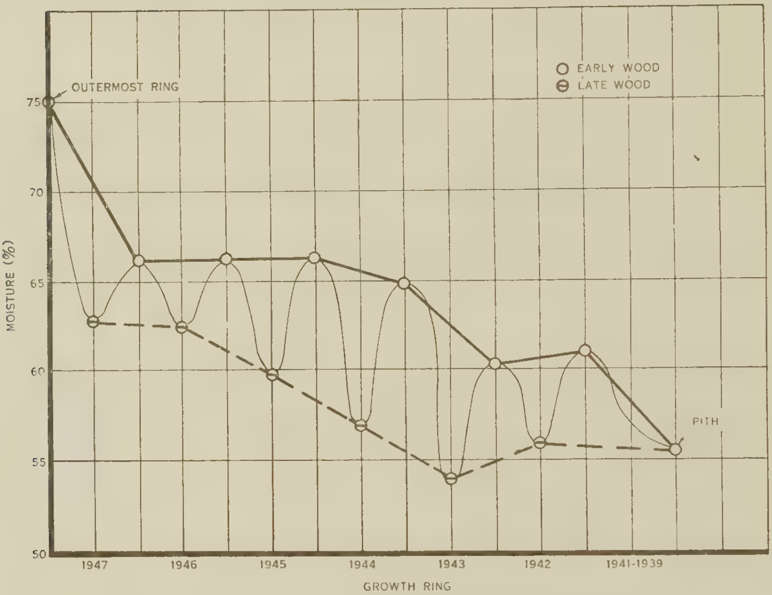

Fig. 1.—*E. regnans*. Breast height water content.

These results can be interpreted on the classical concept of ascending and descending sap streams in the sapwood and phloem respectively, with inward movement of the sap stream through the cambium. The protoplasmic elements of the cambium and developing sapwood retain a higher concentration of inorganic salts than occurs either in the phloem "sap" or sapwood sap. As the developing sapwood matures, its protoplasts deteriorate and their inorganic materials are again yielded up to the ascending sap stream.

The concentrations in the saps of one of the inorganic constituents, viz. chlorides, have been determined (Table 4). Chloride concentration has been expressed in terms of sodium chloride because its molecular weight approximates to the mean of the equivalent weights of other chlorides likely to be present. The protoplasmic areas retain chlorides to some extent, the concentration of chloride being higher in these zones than in others.

Hence, active growth of the cambium takes place in a medium corresponding to an inorganic salt concentration of about 1 per cent., more than half of which is chloride.

The lower limits for inorganic salt concentration based on the 80 per cent. ethanol-extractable substances may be a little too low, because, in the sapwood, chloride is sufficient to account for all the inorganic constituents in the ascending sap stream. It would seem that there must be other inorganic anions there, although they may be in relatively small amounts.

From Tables 3 and 6 it can be seen that the amount of insoluble inorganic constituents in the phloem is relatively high, due to deposition of considerable quantities of calcium oxalate in parenchymatous "crystal cells" (Plate 1, Fig. 4); thus, although the apparent concentration of total inorganic materials in the phloem tissues is higher than in the actively growing areas, most of it is physiologically inert, and the highest concentration of inorganic materials in the sap is in the cambial zone and developing sapwood.

TABLE 3

MINERAL CONSTITUENTS PRESENT IN VARIOUS ZONES OF YOUNG *E. REGNANS* AND OTHER SPECIES

Results expressed on the basis of the dry, fresh sample. (Values in brackets, on the basis of water in the fresh sample)

Zone	Softwoods		Hardwoods		
	Pinus silvestris† (%)	*Picea mariana** (%)	*Fraxinus elatior*† (%)	*Ulmus sativa*† (%)	*Eucalyptus regnans* (%)
Outer "bark"		1·2			2·7 (1·5)
Inner "bark"		3·2			3·9 (2·4)
Cambial zone	6·1 (1·0)	3·6	10·4 (1·4)	20·1 (2·6)	21·5 (1·3)
Developing sapwood ..		0·4	3·9 (1·3)	7·3 (1·7)	3·7 (1·2)
Sapwood	0·2 (0·2)	0·3	0·5 (0·9)	0·5 (0·5)	0·6 (0·4)
Heartwood		0·3			0·2 (0·2)

* Values taken or calculated from the data of Anderson and Pigman (1947).

† Values taken or calculated from the data of Allsopp and Misra (1940).

This non-uniform distribution of concentration within the bole is a chemical indicator of one of the physical factors assisting active growth of the cambial zone against the inwardly directed pressure of the surrounding phloem tissues. The cambium and partly differentiated sapwood elements are able to maintain a higher concentration of solutes and therefore a higher osmotic pressure than the surrounding tissues, a physical condition favouring the vacuolation pressure against the restrictive forces of the phloem.

The micro-incineration photographs provide some confirmation of the analytical data. The ash is seen in Plate 1, Figure 1, to be fairly uniformly distributed through the phloem, fairly densely accumulated in the cambial zone and freshly differentiated elements, and becoming more sparse in the more mature sapwood. Plate 1, Figures 2 and 3, shows higher magnifications of the ash of the cambial zone and adjacent regions.

From the semi-quantitative spectrographic analyses of Table 5, the main elements present are calcium, sodium, potassium, magnesium, and manganese. The higher quantities of iron present in the developing sapwood, sapwood, and heartwood are suspect because steel tools (spokeshave and band saw) were used in preparing the samples. Smaller amounts of phosphorus, aluminium, and boron, and traces of numerous other elements, also occur.

(iii) *Ethanol-soluble Acids and Volatile Esters.*—The ethanol extracts from all zones are acidic (Table 6). The weakest acids are present in the cambial and wood zones, while the strongest acids occur in the outer phloem, possibly as a means of voiding unwanted strong acids with the periodical shedding of the rhytidome.

TABLE 4

CHLORIDES SOLUBLE IN THE 80 PER CENT. ETHANOL EXTRACTS FROM THE ZONES OF YOUNG *E. REGNANS* TREES

All values expressed in terms of sodium chloride

Zone	On Basis of Oven-dried Fresh Sample (%)	On Basis of the Water in the Fresh Sample (%)	On Basis of the Total Soluble Inorganic Constituents (%)
Outer phloem . .	0·4	0·23	44
Inner phloem . .	0·5	0·30	59
Cambial zone . .	11·0	0·68	69
Developing sapwood	1·6	0·54	67
Sapwood	0·2	0·16	100
Heartwood	Negligible	Negligible	—

(iv) *Ethanol-soluble Sugars.*—The materials soluble in 80 per cent. ethanol include substances, other than reducing sugars, which are capable of reducing Fehling's solution and are therefore included as "glucose" in Table 7. However the "glucose" values probably indicate the relative amounts of reducing sugars in the various zones.

Invertase is specific to the hydrolysis of β-D-fructofuranosides, so the sucrose values determined by the invertase inversion are more reliable. Low molecular weight fructosans, if they occur in the nutrient fluids, will be included in the invertase values. Chloride ions may affect the yield of cuprous oxide from Fehling's solution (Van der Plank 1936) but, while such ions will affect the acid inversion values, the invertase inversion values will be almost unaffected because of the low concentration of chloride ions in the ethanol extracts.

Because the ratio between the acid and invertase sucrose values is not constant for all zones, it must be assumed that invertible substances, other than sucrose, are present (especially in the outer phloem).

The data (Table 7) suggest that, in actively growing *E. regnans*, the descending sap stream contains about 2 per cent. of soluble sugars, about three-quarters of which is sucrose. In the cambium and differentiating sapwood the total concentration falls to about half, suggesting that about one-half of the available sugar supply is used for respiration and formation of the primary wall. The almost complete absence of sugars in the sapwood suggests that the remaining half is used for secondary wall formation and condensation (since starch grains have been observed in the rays of the sapwood). The higher glucose/sucrose ratio in the cambium and developing sapwood than in the inner phloem (1/3 in the inner phloem and 1/1 in the cambium) suggests selective utilization of sucrose for cambial activity, either directly, or through a threshold hexose concentration of about 0.4 per cent. after preliminary hydrolysis.

TABLE 5

SEMI-QUANTITATIVE SPECTROGRAPHIC ANALYSIS OF THE ASHES FROM THE ZONES OF *E. REGNANS* *

Element†	Outer Phloem	Inner Phloem	Cambial Zone	Developing Sapwood	Sapwood	Heartwood
Calcium	AP	AP	AP	AP	AP	AP
Sodium	AP	AP	AP	AP	AP	AP
Potassium	AP	AP	AP	AP	AP	AP
Magnesium	AP	AP	AP	P	AP	AP
Manganese	AP	AP	AP	P	P	P
Aluminium	ST	T	P	ST	P	P
Iron	ST	T	T	P	AP	AP
Copper	ST	ST	ST	T	ST	ST
Strontium	T	T	FT	T	ST	ST
Nickel	T	T	T	FT	T	ST
Silver	T	T	T	FT	T	T
Chromium	FT	FT	FT	T	T	T
Lead	T	FT	FT	T	ST	ST
Arsenic	T	T	FT	—	—	—
Zinc	FT	—	FT	FT	T	P
Cadmium	FT	—	—	—	FT	FT
Phosphorus	T	FT	P	P	P	P
Silicon	—	—	FT	T	T	ST
Boron	ST	T	T	ST	P	P
Tin	—	—	—	—	FT	T

* Code: AP = appreciable amount present; P = present; ST = strong trace; T = trace; FT = faint trace; — = not detectable.

† The following elements were also tested for, but were found to be absent: barium, molybdenum, tungsten, vanadium, titanium, cobalt, platinum, bismuth, and antimony.

The ultimate destination of fructose produced by the enzymic hydrolysis of sucrose is open to question. Hibbert (1938) has suggested that lignin may be formed by the condensation of guaiacol (which has been isolated from the sap of certain species—see Schwalbe and Neumann 1930) with a 4-keto-oxypentanal (derived through fructuronic acid from fructose).

Chattaway (unpublished data) has observed that nearly all of the phloem cells in *E. regnans* are alive and that green plastids are present near the outside of the outer phloem. These observations, taken together with the relatively high sugars and protein contents of the outer phloem, suggest that there is photosynthetic activity near the periphery of the phloem.

Although the concentration of sugars in the descending sap stream is given as about 2 per cent., this value is an average sap concentration of the inner phloem, which is not a homogeneous tissue comparable with the cambium. The sap concentration in different elements may be quite different; thus Wislicenus and Hempel (1933) have shown that the sieve tube "sap" of young *Quercus* spp. contains sucrose, glucose, and fructose in concentrations of approximately 14.0, 0.4, and 0.2 per cent. respectively. This is a much higher sugar concentration than the average value of 2 per cent. found in this study, but it is possible that the sieve tube concentration would be much higher.

Table 6

MINERAL CONSTITUENTS, ACIDS, AND VOLATILE ESTERS PRESENT IN THE 80 PER CENT. ETHANOL EXTRACTS FROM THE ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

Zone	Mineral Constituents on the Basis of the Total Mineral Constituents (%)	pH of 80 per cent. Ethanol Extract	Acids and Esters (equivalents $\times 10^4$) on the Basis of the Water in the Fresh Sample		
			Volatile Free Acids (%)	Non-volatile Free Acids (%)	Volatile Esters (%)
Outer phloem ..	33	4·1	4	200	20
Inner phloem ..	22	5·7	2	100	40
Cambial zone ..	74	6·5	0·4	20	0·05
Developing sapwood	66	6·3	1	20	—
Sapwood	45	6·2	Nil	10	Nil
Heartwood ..	30	6·5			

Allsopp and Misra (1940) stated that "the sugar content of the new wood is much lower than that of the cambium" in ash and elm. If sugar concentrations are calculated from their data, it is found that they decrease in the new wood to about 60 per cent. of that of the cambium, which is not such a great decrease as Allsopp and Misra imply. In *E. regnans* the protoplasts of the wood elements have been found to be living some distance in from the cambium and the analyses in Table 7 show that the sugar concentration in the new wood (developing sapwood) is about the same as is found in the cambium. Therefore the depletion of sugars to very low values does not occur until the protoplasts of the wood elements deteriorate.

The more or less constant ratio between total sugars and 80 per cent. ethanol solubles (4(*b*) in Table 7), in the inner phloem, cambial, and developing sapwood zones, is of interest: it may indicate that the 80 per cent. ethanol-soluble nutrient materials are assimilated at the same rate during cell formation and cell growth.

Table 7

REDUCING AND INVERTIBLE SUBSTANCES SOLUBLE IN 80 PER CENT. ETHANOL FROM THE VARIOUS ZONES OF YOUNG *E. REGNANS* TREES

Determination	Basis*	Zone					
		Outer Phloem	Inner Phloem	Cambial Zone	Developing Sapwood	Sapwood	Heartwood
(1) Reducing substances calculated as glucose	(*a*)	1·2	0·9	6·3	1·9	Negligible	Negligible
	(*b*)	10·1	8·3	14·2	19·5	—	—
	(*c*)	0·7	0·5	0·4	0·6	—	—
(2) Invertible substances calculated as sucrose (acid inversion)	(*a*)	0·8	3·0	6·9	1·5	Negligible	Negligible
	(*b*)	6·7	28·0	15·7	15·4	—	—
	(*c*)	0·5	1·8	0·4	0·5	—	—
(3) Invertible substances calculated as sucrose (invertase inversion)	(*a*)	0·5	2·4	6·6	1·2	Negligible	Negligible
	(*b*)	4·4	23·0	14·9	12·3	—	—
	(*c*)	0·3	1·5	0·4	0·4	—	—
(4) Total "sugars", i.e., (1)+(3)	(*a*)	1·7	3·3	12·9	3·1	Negligible	Negligible
	(*b*)	14·5	31·3	29·1	31·8	—	—
	(*c*)	1·0	2·0	0·8	1·0	—	—

* All results are expressed as percentages of:
(*a*) the dry, fresh samples;
(*b*) the 80 per cent. ethanol solubles; and
(*c*) the quantity of water in the original fresh samples.

(v) *Tannin and Kino.*—From Table 8 it may be observed that oven drying at 105°C. has little apparent effect on the water-solubles determination.

Table 9 shows that the 80 per cent. ethanol-soluble tannin is not included, except perhaps in small amounts, in the tannin extracted from the inner and outer phloems by water, and that both water-soluble and ethanol-soluble tannins occur in these tissues. It is probable that column 6, Table 8, includes the tannin listed in column 1 of Table 9. Hence the difference represents the non-tannin 80 per cent. ethanol-soluble material in each zone. This is greatest in the inner phloem, which may suggest lignin precursors in this region. The absence of appreciable amounts of water-soluble tannin, in all but the phloem of the tree, is noteworthy.

The tannin contents of the various zones (column 1, Table 9) appear to rise from a minimum in the sapwood to a maximum in the outer phloem. The high tannin content of the heartwood is suggestive of either infiltration or increased metabolic activity of the cells during sapwood-heartwood transition.

When the 80 per cent. ethanol-soluble tannin is calculated on the basis of either the original sap solution or the 80 per cent. ethanol solubles in each zone, a slightly different picture is obtained. In this case the tannin is present in minimal amounts in the cambium and increases on either side of the cambium. This may indicate that kino and tannin are waste materials excreted to either side of the cambium during metabolism and eventually accumulated in the tanniniferous cells of the phloem and the dead cells of the heartwood. In the sapwood-heartwood transition area the ray cells become very active before their death. The content of tannin-like materials in the heartwood may be augmented by by-products of this burst of ray cell activity. Furthermore, since kino, tannin, and lignin are structurally based on aromatic nuclei it is probable that kino and tannin are by-products formed during the plant's synthesis of lignin.

TABLE 8

MATERIAL SOLUBILIZED, BY HOT WATER AND BY HOT 80 PER CENT., ETHANOL, FROM THE ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

All results expressed on the basis of the dry, fresh sample

Zone	Material Soluble in Water at 95°C.			Material Soluble in Boiling 80 per cent. Ethanol (%)	Total Soluble Material, i.e. (4)+(3) (%)	Material Soluble in 80 per cent. Ethanol, but Insoluble in Water, i.e. (5)−(2) (%)
	Oven-dried Sample (%)	Air-dried Sample (%)	Ethanol-extracted Sample (%)			
	(1)	(2)	(3)	(4)	(5)	(6)
Outer phloem	20·2	20·6	12·5	11·5	24	3·4
Inner phloem	13·0	13·3	10·5	10·4	21	7·6
Cambial zone			14·8	44·5	59	
Developing sapwood	12·5	12·2	3·6	9·6	13	0·8
Sapwood		2·6	1·2	1·6	3	0·2
Heartwood		2·5	1·3	2·7	4	1·5

(*b*) *Nitrogenous Substances*

Analyses of the air-dried, oven-dried, and 80 per cent. ethanol-extracted zonal samples have been made for total nitrogen, ammoniacal nitrogen, and nitrate nitrogen. After making due allowance for the last two forms of nitrogen, the protein has been calculated by using the factor 6.4.

Most proteins, other than the prolamine type, are rendered insoluble on treatment with boiling 80 per cent. ethanol. However, small amounts of nitrogen are present in the 80 per cent. ethanol extracts (Table 1). Part may be derived from hydrolytic degradation of proteins through mild ethanolysis, but it is more probable that most of the soluble nitrogen is contained in compounds such as amino acids and inorganic nitrates.

As would be expected, proteins and nitrates are particularly plentiful in the cambial-developing sapwood region (Table 10). When expressed on the basis of the dry zonal substances, the nitrate content of the cambial region appears unduly high, but not when expressed on the basis of the sap solution, viz.:

Inner phloem	0.06 per cent.
Cambial zone	0.07 per cent.
Developing sapwood	0.07 per cent.

However, because nitrates are usually soluble and because of the low nitrogen content of the 80 per cent. ethanol solubles (Table 1), it is possible that protein degradation products may liberate ammonia, which is liberated quantitatively by nitrates, under the conditions of the nitrate determination. Thus the nitrate values in Table 10 are probably too high.

TABLE 9

TANNIN-LIKE MATERIALS, SOLUBILIZED BY 80 PER CENT. ETHANOL AND BY HOT WATER, FROM THE ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

Zone	Tannins Soluble in Hot 80 per cent. Ethanol			Tannins Soluble in Water at 95°C.	
	On Basis of the Dry, Fresh Sample (%)	On Basis of the Water in the Original Fresh Sample (%)	On Basis of the 80 per cent. Ethanol Solubles (%)	Extracted from the Oven-dried Sample and on the Basis of the Dry, Fresh Sample (%)	Extracted from the 80 per cent. Ethanol-extracted Sample and on the Basis of the Dry, Fresh Sample (%)
	(1)	(2)	(3)	(4)	(5)
Outer phloem	2·3	1·2	20	5·2	5·5
Inner phloem	1·7	0·9	16	1·5	1·8
Cambial zone	0·4	0·02	0·8	—	—
Developing sapwood	0·1	0·04	1·1	Negligible	Negligible
Sapwood	0·04	0·02	3	Negligible	Negligible
Heartwood	0·4	0·3	16	Negligible	Negligible

The high protein content of the phloem, and the relatively high concentration of inorganic solutes in the phloem fluids, indicate protoplasmic activity in the cells of this region.

The amounts of ammonia, or rather of ammonium salts, are very low in all zones.

(*c*) *Wood Substance*

(i) *Lignin-like Constituents.*—Lignin was determined by the Klason method without any alkaline pretreatment, because the effect of an alkaline treatment on the constituents of the partly differentiated and freshly differentiated tissues is unknown. Therefore the Klason lignin values should be regarded as apparent, rather than true, lignin values.

From the results in Table 11 it is evident that the method of drying affects the apparent Klason lignin content, particularly in the phloem region. On heating the samples at 105°C. for some hours, certain heat-labile or readily oxidizable constituents are apparently altered and become insoluble on subsequent Klason lignin treatment. Very little may be said concerning the nature of these labile constituents except that they contain no, or very few, methoxyl groups: thus the differences between the methoxyl contents of the Klason lignins from the oven-dried and air-dried samples from each zone are chiefly accounted for by the observed yields of Klason lignin.

Table 10

PROTEIN, AMMONIUM SALTS, AND NITRATES PRESENT IN THE ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

All results expressed on the basis of the dry, fresh sample

Zone	Protein (factor 6·4)			Nitrate Ion (calculated as sodium nitrate)	Ammonium Ion (calculated as ammonia)	
	Ethanol-extracted Sample (%)	Oven-dried Sample (%)	Air-dried Sample (%)	Oven-dried Sample (%)	Ethanol-extracted Sample (%)	Air-dried Sample (%)
Outer phloem ..	2·7	2·8	2·9	0·5	0·02	0·03
Inner phloem ..	1·8	1·9	1·9	0·1	0·02	0·03
Cambial zone ..	12·5	13·1	—	1·2	0·02	—
Developing sapwood	2·8	3·6	3·3	0·2	0·02	0·03
Sapwood	0·6		0·4	0·1		0·03
Heartwood	0·3		0·2	0·1		0·01

The Klason lignins from the 80 per cent. ethanol extracts of the cambial and developing sapwood zones contained much waxy material. This material, together with protein and carbohydrate degradation products and possibly low-methoxyl lignin precursors, may account for the high apparent lignin content of the cambial zone. The values in column 5, Table 11, agree fairly well with the values in columns 1 and 2. The low methoxyl contents, especially in the phloem, of the soluble "lignin" are probably due to the inclusion of kino and tannin. The lignin values of 0.7 and 1.5 per cent. (with a methoxyl content of 12 per cent.) for the sapwood and heartwood respectively would indicate the presence of small quantities of soluble "native" lignin in these zones.

If it is assumed that the Klason lignin from *E. regnans* has a methoxyl content of about 22.6 per cent. then, in all outer zones, either the material estimated as Klason lignin is very different from the lignin in mature wood, or alternatively the isolated Klason lignin is a mixture of true Klason lignin with artifacts formed during the isolation process. The phloem fractions contain cells rich in substances that have been observed, in microscopic sections (Plate 2, Fig. 3), to develop into insoluble materials on treatment with 72 per cent. sulphuric acid; these materials, which have been designated as "kino bodies," remain as yellow aggregates. Microscopic examination of the various Klason lignins from the several zones has shown that, in the outer phloem, such aggregates are still visible. Further, there is evidence of the presence of fibrous matter and residual cell wall material (Plate 2, Fig. 4).

TABLE 11

RESULTS OF KLASON LIGNIN AND METHOXYL-IN-LIGNIN DETERMINATIONS ON THE VARIOUS ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

All results, except those in brackets, are expressed on the basis of the dry, fresh samples. Methoxyl values are given enclosed in brackets and are expressed on the basis of the dry Klason lignins

Zone	Oven-dried Sample (%)	Air-dried Sample (%)	Ethanol-extracted Sample (%)	Ethanol Extract (%)	Sum i.e. (3)+(4) (%)
	(1)	(2)	(3)	(4)	(5)
Outer phloem	31·4 (6·4)	25·3 (7·4)	25·0 (7·8)	4·2 (2·7)	29·2
Inner phloem	21·8 (12·1)	20·4 (13·3)	18·5 (13·5)	2·0 (2·2)	20·5
Cambial zone*	19·0 (1·3)	— —	11·9 (1·4)	5·4	17·3
Developing sapwood* ..	12·2 (14·5)	10·8 (15·9)	11·2 (17·4)	1·0	12·2
Sapwood	22·6	22·4 (22·6)	21·6	0·7 (12·2)	22·3
Heartwood	25·5	25·2 (22·2)	23·6	1·5 (12·2)	25·1

* The evolution of hydrochloric acid gas was very noticeable during the Klason lignin determinations on these zones.

Table 12 presents the yields of aldehydes, and their partial compositions, obtained by the alkaline nitrobenzene oxidation of inner phloem, outer phloem, sapwood, and heartwood. This treatment converts a portion of the constituent basic units of lignin to low-molecular-weight aldehydes, viz. vanillin and syringaldehyde. The ratio of vanillin to syringaldehyde depends upon the species of wood used, the ratio being high in softwoods and of the order of 0.2-0.4 for hardwoods. It has been shown by Bland, Ho, and Cohen (1950) that the ratio is of the order of 0.25 for some eucalypt samples.

All four zones give approximately 10 per cent. yields of mixed aldehydes (Table 12); however, the sum of vanillin and syringaldehyde from the phloem lignins amounts to only about one-half that from the mature wood lignins. The other aldehydes (volatile and non-volatile) therefore amount to about 55 per cent. from the phloems and to only 14 per cent. from the mature wood. As the ratio of vanillin to syringaldehyde is of the same order in all cases, it would appear that there is an additional source of appreciable amounts of aldehydes in the phloem tissues. It is unlikely that the additional aldehydes are derived from kino because Bland, Ho, and Cohen (1950) have shown that, in *Eucalyptus marginata* Sm., which contains large amounts of alkali-soluble cellular kino, an alkaline pretreatment caused little alteration to the ratio of vanillin to syringaldehyde or to the yield of mixed aldehydes.

TABLE 12

RESULTS OF ALKALINE NITROBENZENE OXIDATIONS OF SEVERAL ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES, TOGETHER WITH THE CALCULATED "TRUE" LIGNIN CONTENTS OF THE VARIOUS ZONES

Zone	Observed Klason Lignin on the Basis of Dry, Fresh Sample (%)	Methoxyl Content of the Klason Lignin (%)	Yield of Aldehydes on the Basis of Dry, Fresh Sample (*A*%)	Vanillin (V) (% of *A*)	Syring-aldehyde (S) (% of *A*)	V/S	Non-volatile Aldehydes (% of *A*)	Volatile Aldehydes Other Than V or S (% of *A*)	Calculated "True" Lignin Content on the Basis of the Dry, Fresh Sample		
									Method I (%)	Method II (%)	Method III (%)
Outer phloem	25·3	7·4	10·0	7·3	39·4	0·22	29·7	23·6	13·7	8·3	8·4
Inner phloem	20·4	13·3	10·0	4·8	39·1	0·15	38·4	17·6	12·8	12·0	11·6
Cambial zone	19·0	1·3								1·1	0·0
Developing sapwood ..	10·8	15·9								7·6	11·5
Sapwood*	22·5	22·6	9·2	14·5	68·7	0·25	10·8	5·6	22·5	22·5	25·4
Heartwood*	23·9	22·6	7·9	14·7	73·6	0·24	4·6	7·0	20·5	23·9	26·4

* These data were obtained from samples of young *E. regnans* other than those used for the zonal investigations; however, the samples were of the same age and from the same locality.

Furthermore, the data presented provide several methods for calculating the actual percentages of lignin in the various zones. For methods I and II (Table 12) it is necessary to assume that the amount of Klason lignin determined in the sapwood represents the actual amount of lignin in this zone; the actual lignin in the other fractions is of similar chemical composition to that in the sapwood (this assumption is supported by the vanillin/syringaldehyde ratio being of the same order for the phloem and mature wood samples); and the methoxyl content of Klason lignin is about 22.6 per cent. The sapwood has been taken as the standard for comparison because the lignin in this zone is freshly laid down, whereas that in the heartwood has "aged" for several years and may have become chemically modified during this process. In addition certain 72 per cent. sulphuric acid insoluble infiltration products are probably present in the heartwood.

The values obtained by method I were calculated from the sum of the vanillin and syringaldehyde yields for each zone using, as a basis, the Klason lignin yield and the sum of the vanillin and syringaldehyde yields of the sapwood. The values obtained by method II were calculated from the yield and methoxyl content of the Klason lignin from each zone using, as a basis, the yield and methoxyl content of the Klason lignin from the sapwood. For the calculation of the native lignin contents (method III, Table 12), it has been assumed that each uronic acid residue contains one methoxyl group attached to it; the remaining methoxyl groups are attached to lignin; and the native lignin contains about 25 per cent. methoxyl.

The various calculated lignin contents, for each zone, agree reasonably well, and may be assumed to give an approximate value for the amount of lignin in the phloem and in the developing wood fractions. Thus the indications from the calculated data present a very different picture from those determined experimentally, and confirm microscopic staining evidence on the way in which lignin is deposited.

The results in Tables 11 and 12 suggest either (*a*) that there is no lignification in the cambial zone and that lignification commences in the developing sapwood and is completed in the sapwood; or (*b*) that if "lignification" commences in the cambial zone, then "lignin" must be derived from substances that contain few or no methoxyl groups. If (*b*) is correct, then the "lignin" must be methylated in the developing sapwood-sapwood region. This, although possible, does not appear probable because the apparent Klason lignin content of the developing sapwood is lower than that of the cambial region.

(ii) *Carbohydrate Constituents.*—It is probable that cellulose is present in the cambial region of *E. regnans* trees because, for example, Preston and Wardrop (1949) have shown that micellar cellulose is present in the cambial cells of *Pinus silvestris* L. However, it is evident, from the calculated hexose carbohydrate* values in Table 13, that the amount of cellulose in the cambial zone cells cannot exceed 22 per cent. of the ethanol-extracted material (or 12 per cent. of the unextracted material).

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* Because the "hexose carbohydrate" values have been calculated from the holocellulose yields, the values will be too high and will include such constituents as residual lignin, methoxyl due to uronic acid groups, etc. The hexose carbohydrate values will include all cellulose as well as non-resistant hexose polymers.

The holocellulose values for the developing sapwood indicate that the deposition of carbohydrates is essentially complete in this zone, for the value, obtained on calculating the holocellulose content of the developing sapwood on the basis of the lignin content of the sapwood, is close to that of the holocellulose content of the sapwood. The above interpretation of the analytical results is probably correct because Dadswell and Griffin (unpublished data) have shown that the complete differentiation of the wood fibre in eucalypts takes approximately 2 weeks and it is during this relatively short time that the cellulose is laid down. The maximum rate of cambial cell division has been shown to be approximately three divisions in 2 days in *Eucalyptus gigantea* Hook f. (Amos, Bisset, and Dadswell 1950). There are, therefore, approximately 20 differentiating cells per cambial initial at the time of maximum growth rate within which the protoplasts are manufacturing and laying down cellulose upon the initial primary wall.

It will be noted, from Table 13, that the percentages of most carbohydrate constituents are higher in the developing sapwood and heartwood than in the sapwood. The difference, in the developing sapwood and sapwood, is reduced when the values for the carbohydrate constituents are expressed on a lignin-free basis. On this basis the sapwood contains greater amounts of certain constituents, for example, pentosan. Variation between the analyses of the sapwood and developing sapwood may be expected because the sapwood is a mixture of early wood and late wood, whereas the developing sapwood is largely early wood. Ritter and Fleck (1926) have shown that, for several timber species, there are differences in chemical properties between early wood and late wood; for example, pentosan is higher and cellulose is lower in early wood than in late wood.

The higher percentages of pentosan, uronic acid, and galactose in the heartwood, as compared with the sapwood, are more difficult to explain. It is possible that, because the zones were laid down at different times, differences are due to varying growth conditions influencing the products of secondary growth. The senescent metabolism of the ray cells during sapwood-heartwood transition might also enhance the proportion of some of the carbohydrate constituents in the heartwood. Again, chemical variation may accompany morphological changes from the pith outwards; thus Watson *et al.* (1952) have shown that the further a growth ring is from the pith of *Pinus radiata* D. Don, the higher is the pulp yield and the lower the pentosan content, lignin remaining practically constant. If the like applies to hardwoods, the early-formed wood of the tree (i.e. the wood that was heartwood at the time of sampling) would be generally higher in pentosan (and hence in hemicellulose) and lower in pulp yield (and hence in cellulose) than that formed in later years. Bisset and Dadswell (1949) showed that in *E. regnans* "at all levels there was a rapid increase in length of both early and late wood fibres from the centre of the tree outwards for approximately 10 growth rings, after which a more or less constant value was reached in each case." Hence, morphological changes similar to those in softwoods do occur in hardwoods and it remains to be seen whether similar chemical changes also occur.

The uronic acid content of the phloem zones and of the cambial zone is considerably higher than that of the wood zones (Table 13). Evidence from staining with ruthenium red, a stain fixed by the carboxyl groups of pectic acid and probably other uronic-acid-containing substances (Joslyn and Phaff 1947), also indicates that these materials are plentiful in the sieve tubes of the phloem, ray cells, and undifferentiated cell walls.

The galactose values, listed in Table 13, do not distinguish between galactose and galacturonic acid but include both of these carbohydrate constituents. Therefore, because pectin consists chiefly of galacturonic acid residues, it may be concluded that not more than 15 per cent. of the uronic acid content of the cambial zone is due to pectinous material. The pectin content of this zone may be much less than the 1.5 per cent. shown in Table 13, because it has been shown by one of the authors (C.M.S., unpublished data) that the non-resistant carbohydrates of *E. regnans* wood contain measurable quantities of galactose. The existence of pectin in some woods has been rendered doubtful by the work of Jensen and Nuotio (1951) who have shown that the wood of *Betula verrucosa* Ehrh. contains no galactose (and hence no galacturonic acid or pectin): however, the inaccuracies of Schorger's method for galactose determination are such that galactose, equivalent to a few tenths of 1 per cent. of the weight of the original wood, may have remained undetected.

The high uronic acid, low pentosan, high galactose, and low acetyl contents of the phloem zones, when compared with the wood zones, indicate that considerable differences exist between the composition of the phloem and of the wood hemicelluloses: the molar ratio (Table 13) of pentosan/uronic acid anhydride emphasizes the difference, because the ratio is about 2/1 for the phloem zones and about 6/1 for the wood zones. The phloem of *E. regnans* (Plate 2, Fig. 1) contains fewer fibres than the xylem, being largely sieve tubes and parenchyma cells (ray, crystalliferous, and tanniniferous). The hemicelluloses of wood and phloem fibres may have similar compositions. Harlow and Wise (1928) have shown that there are marked differences in chemical composition between the rays and whole wood of *Casuarina inophloia* F. Muell. et Bail. and of *Quercus alba* L. Thus the difference observed may be due to a difference between the composition of the hemicelluloses of the parenchyma and fibre cells.

The considerably lower hexose carbohydrate content of the outer phloem, as compared with that of the inner phloem, is of interest. Chattaway (unpublished data) has shown that the cells of the phloem of *E. regnans* are mostly living and that the parenchyma cell walls become thicker with distance outwards from the cambium. Thus it would be expected that the hexose carbohydrate content of the outer phloem would be greater than that of the inner phloem. The observed decrease in hexose carbohydrate, therefore, may be attributed to infiltration of kino and other substances into the outer phloem.

Table 13

RESULTS OF ANALYSES FOR SEVERAL CARBOHYDRATE CONSTITUENTS ON THE ZONAL SAMPLES OF YOUNG *E. REGNANS* TREES

All results, except where otherwise indicated, are expressed on the basis of the dry weight of sample taken for analysis.

Determination	Sample	Zone					
		Outer Phloem (%)	Inner Phloem (%)	Cambial Zone (%)	Developing Sapwood (%)	Sapwood (%)	Heartwood (%)
(1) Galactose	(*a*) Oven-dried at 105° C.						
	(*b*) Air-dried	3·0	2·9		1·2	0·9	
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)			0·8			1·3
	(*d*) 80% Ethanol-extracted			1·5			1·3
(2) Uronic acid anhydride (using method of Dickson, Otterson, and Link 1930)	(*a*) Oven-dried at 105° C.	10·8	8·6		7·4		
	(*b*) Air-dried	10·3	8·6		7·4	5·8	6·4
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	8·7	7·1	8·0	5·4	4·5	5·6
	(*d*) 80% Ethanol-extracted	9·8	7·9	14·4	6·0	4·7	5·8
(3) Uronic acid anhydride (using method of Whistler, Martin, and Harris 1940)	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	7·7	5·4	6·0	4·3	3·2	4·2
	(*d*) 80% Ethanol-extracted	8·7	6·0	10·9	4·8	3·3	4·3
(4) Ratio, 2(*c*)/3(*c*)		1·12	1·32	1·32	1·25	1·42	1·34
(5) Total pentosan	(*a*) Oven-dried at 105° C.	13·9	12·7	7·9	19·8		
	(*b*) Air-dried	14·0	13·0		18·6		19·4
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	14·1	12·5	7·8	18·9	17·0	19·4
	(*d*) 80% Ethanol-extracted	16·0	13·9	14·1	20·9	17·3	19·9
(6) Pentosan, corrected for furfural from uronic acid	(*a*) Oven-dried at 105° C.	11·2	10·6	5·9	17·9		
	(*b*) Air-dried	11·3	10·9		16·8		17·8
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	11·6	10·7	5·8	17·4	16·1	17·9
	(*d*) 80% Ethanol-extracted	13·1	11·9	10·5	19·3	16·3	18·4

TABLE 13 (*Continued*)

Determination	Sample	Zone					
		Outer Phloem (%)	Inner Phloem (%)	Cambial Zone (%)	Developing Sapwood (%)	Sapwood (%)	Heartwood (%)
(7) Molar ratio, 6(*c*)/3(*c*)		2·0	2·6	1·3	5·3	6·6	5·7
(8) Acetyl	(*a*) Oven-dried at 105° C.			3·1	3·6	3·7	4·3
	(*b*) Air-dried	2·4	2·6		3·6	3·7	4·3
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	2·5	2·6	3·0	3·8	3·6	4·4
	(*d*) 80% Ethanol-extracted	2·8	2·8	5·4	4·2	3·6	4·5
(9) Hollocellulose (chlorite)	(*a*) Oven-dried at 105° C.			31·3*			
	(*b*) Air-dried	66·4			82·1		
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	63·1	72·0	33·4* (27†)	81·9	83·8	83·9
	(*d*) 80% Ethanol-extracted	71·4	80·0	60·1* (49†)	90·6	85·2	86·0
(10) Hexose carbohydrate (i.e. 9−(3+6+8))	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	41	53	18* (12†)	54	59	58
	(*d*) 80% Ethanol-extracted	46	59	33* (22†)	60	60	60
(11) Methoxyl	(*a*) Oven-dried at 105° C.	3·6	3·9	0·9	3·5		
	(*b*) Air-dried	3·5	4·2		3·7	7·2	7·6
	(*c*) 80% Ethanol-extracted (dry, fresh sample basis)	3·4	3·8	0·9	3·6	6·9	7·3
	(*d*) 80% Ethanol-extracted	3·8	4·3	1·6	4·0	7·0	7·5

* The cambial zone holocellulose samples contain appreciable quantities of nitrogenous material.

† These values have been corrected for the protein content of the holocellulose.

This explanation is supported by the high water-solubles, tannin, and apparent Klason lignin contents as well as by the low methoxyl content of the outer phloem.

V. Acknowledgments

The authors wish to acknowledge the encouragement and criticism of Dr. W. E. Cohen and Dr. H. E. Dadswell during the course of this work.

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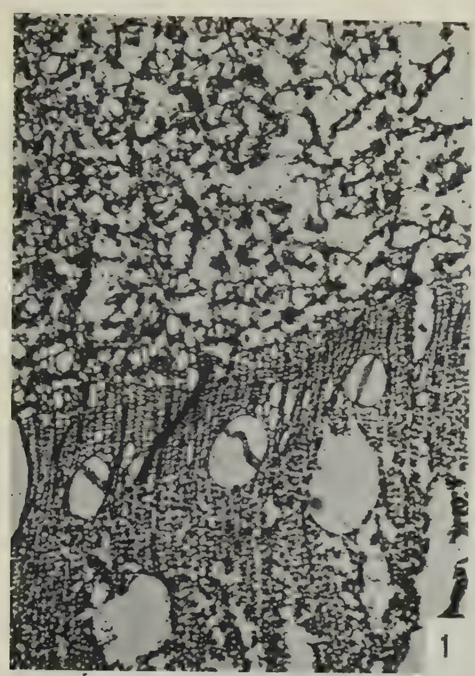

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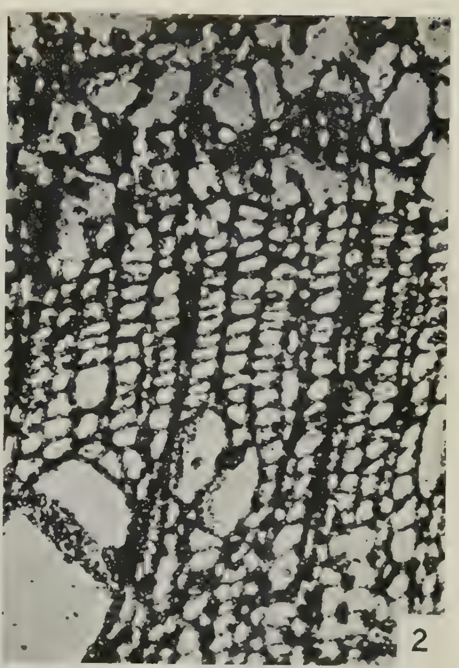

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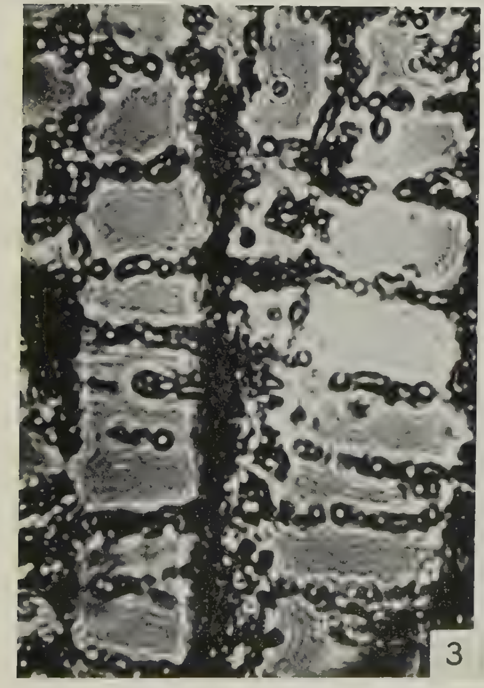

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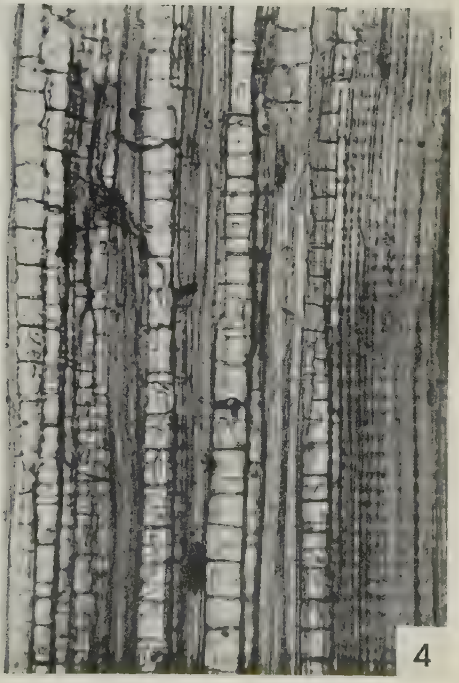

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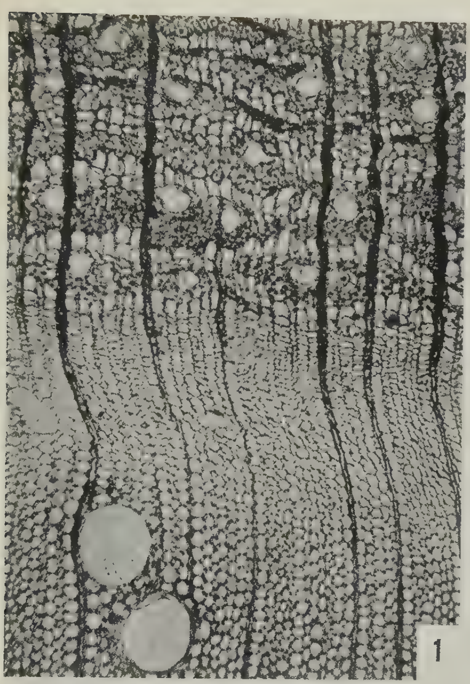

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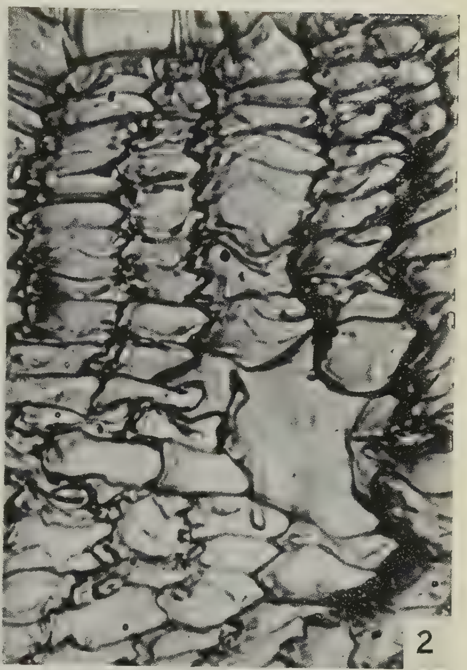

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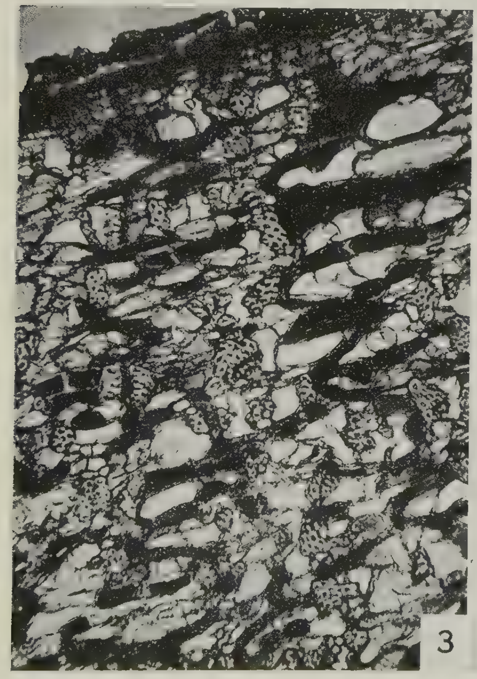

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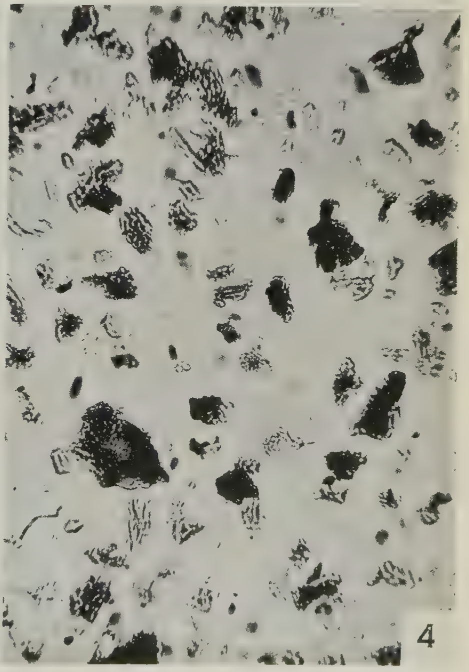

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Explanation of Plates 1 and 2

Plate 1

Fig. 1.—Micro-incineration of cross section of cambium, phloem, and wood of *E. regnans*. × 50. Note high concentration of mineral matter in cambial zone.

Fig. 2.—Micro-incineration of cross section of cambial zone of *E. regnans*. × 200.

Fig. 3.—Micro-incineration of cross section of cambial zone of *E. regnans*. × 1100.

Fig. 4.—Longitudinal radial section of *E. regnans* showing cambial zone and newly differentiated crystal cells with crystals fully formed. × 180.

Plate 2

Fig. 1.—Transverse section of inner phloem, cambial zone, and developing sapwood of *E. regnans*. × 90. Note small amount of structural material in cambial zone.

Fig. 2.—Transverse section of cambial zone of *E. regnans*. × 1100. Note thin walls with little structural material.

Fig. 3.—Transverse section of phloem of *E. regnans* showing cells which stain deeply after contact with the microtome knife and which produce "kino bodies" with 72 per cent. sulphuric acid. × 90.

Fig. 4.—Klason lignin from phloem of *E. regnans* showing some fibrous and cellular structures remaining. × 260.

FUNGAL CELLULASES

III. *STACHYBOTRYS ATRA*: GROWTH AND ENZYME PRODUCTION ON NON-CELLULOSIC SUBSTRATES

By M. A. JERMYN*

[*Manuscript received October* 6, 1952]

Summary

The growth of the mould *Stachybotrys atra* on modified Waksman-Carey medium has been studied. Iron, zinc, copper, and manganese have been shown to be inorganic micronutrients for the mould but their essential nature has not been conclusively demonstrated. Certain other supplements appear to stimulate growth.

When ammonia is the nitrogen source, the mould makes good growth only with sugars and related compounds as carbon sources; the only other carbon source readily utilized is acetate. Replacement of ammonia by nitrate assists the utilization of a range of organic acids. A wide range of nitrogen sources can be utilized for growth; proteins are good sources of both carbon and nitrogen, but individual amino acids are inadequate.

The course of growth on sucrose, starch, and both buffered and unbuffered glucose media has been followed. Enzymes with β-glucosidase activities were produced on all media and the presence of substrates containing β-glucosidic linkages was unnecessary for the production of activity simulating the "C_x enzyme" (enzyme splitting polymeric β-glucosidic linkages) of Reese, Siu, and Levinson (1949).

High activity for the hydrolysis of salicin and sodium carboxymethyl cellulose appears in the medium at and just after the germination of the mould. In unbuffered media a second maximum is associated with the attainment of maximum mycelial weight. Enzymic activity against *p*-nitrophenyl-β-glucoside does not appear to be closely correlated with the other two activities. The meaning of these results is discussed in terms of the hypothesis of multiple β-glucosidase activities and the non-existence of a specific "C_x" enzyme (Jermyn 1952*b*).

I. INTRODUCTION

Stachybotrys atra Corda is typically north temperate in its distribution according to White, Yeager, and Shotts (1949) but is also a very frequent isolate in the Melbourne area (Cox *et al.* 1945). In tropical areas S. *atra* is replaced by the very closely related *Memnoniella echinata* (Riv.) Gallow. The complete elimination of invalid species in *Stachybotrys* has not yet been accomplished and Bisby (1945) believes that many of the species described will eventually be reduced to synonymy in the highly variable S. *atra*. The uncertainty about identifications in this genus must be borne in mind in comparing the various physiological studies in the literature. Siu (1951) classifies S. *atra* among the "strong" cellulolytic fungi with abundant isolations from decomposing cellulosic

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materials in the field; the "very strong" *Myrothecium verrucaria* on which the majority of studies on fungal cellulase have been made in the past few years (Saunders, Siu, and Genest 1948; Whitaker 1951) seems unable to maintain itself on cellulosic substrates in the field. For this reason S. *atra* has been selected for fundamental studies in an Australian laboratory on the production of cellulolytic enzymes by microorganisms.

Reese, Siu, and Levinson (1949) have postulated that two types of enzymes are required for the breakdown of cellulose. The first ("C_1 enzyme"), which is present only in cellulolytic fungi, degrades native cellulose to a form that can be further hydrolysed by the second ("C_x enzyme") which is present in all cellulolytic and most non-cellulolytic fungi. It has been shown (Jermyn 1952*a*, 1952*b*) that the non-cellulolytic fungus *Aspergillus oryzae* releases C_x activity into the culture medium and that this activity is due to the presence of some eight β-glucosidases, which have different degrees of specificity for different substrates. Production of these enzymes took place in the absence from the culture medium of substrates having β-glucosidic linkages.

The work presented in this paper is designed to test whether the "C_x" activity of S. *atra* is produced in the culture medium in the absence of β-glucosidic carbon sources and to discover conditions for producing the enzyme or enzymes involved in this activity in sufficient concentrations to warrant purification. It also extends the results of Perlman (1948) to give a wide enough preliminary survey of the growth of S. *atra* in shake culture to serve as a basis for a programme of physiological research on the fungus.

II. Cultural Conditions

To simplify this preliminary study only certain of the possible variables in culture conditions have been investigated. Except where specific mention of the fact is made, the conditions specified in the description below were used throughout.

Incubation was carried out in a warm room maintained at 28 ± 0.5°C., which is approximately the optimum temperature for growth of S. *atra* (Basu 1948). The shaker used was adapted from the design of Jennison *et al.* (1950); it had a reciprocating action with a total excursion of 2 in. and a speed of 96 cycles per minute. Where a continuous record of the pH of the culture medium was to be taken, a Leeds and Northrup Micromax recording pH meter with shielded leads was used. It was found that the directly determined pH of the culture medium and that recorded on the chart were in agreement for runs of up to 3 weeks.

A culture of S. *atra* was obtained from Mr. F. M. Crook of the Defence Research Laboratories, Melbourne. It agreed in all particulars with the literature descriptions of the species. The fungus was maintained by serial transfer on potato-dextrose sugar slopes. After 10 days growth at 30°C. tubes were kept in a refrigerator at 5°C. till required. A spore suspension from the tubes was used to inoculate a layer of potato-dextrose agar medium in 250 ml. Roux bottles. After 10-14 days incubation at 28°C., the contents of the Roux bottle were used to prepare spore suspensions for inoculating the culture flasks.

The basic mineral medium used throughout was that of Waksman and Carey (1926) as modified by Fahraeus (1947). In accordance with the findings of Marsh and Bollenbacher (1946), Perlman (1948), and Buston and Basu (1948), 12 μg. of biotin was routinely added per litre of medium. After experiments on mineral nutrition had indicated the stimulating effect of zinc ions, the Waksman-Carey medium was further modified by the addition of 2 mg./l. of $ZnSO_4.7H_2O$. The final composition of the medium was as follows:

	g./l.
$(NH_4)_2HPO_4$	2.5
$MgSO_4.7H_2O$	0.5
KCl	0.5
$CaCl_2$	20×10^{-3}
$FeSO_4.7H_2O$	10×10^{-3}
$ZnSO_4.7H_2O$	2×10^{-3}
$MnSO_4.4H_2O$	1×10^{-3}
D-biotin	12×10^{-6}

Using this medium experiments were performed to test:

(i) *Ability of* S. atra *to Use Various Carbon and Nitrogen Sources.*—The medium was distributed into 250-ml. conical flasks, 50 ml. being added per flask and, after autoclaving, 0.2 ml. of spore suspension containing 10^9 spores/ml. was added to each.

(ii) *Enzyme Production by* S. atra *on Various Media.*—A three-necked, 2-l. Woulffe bottle containing 500 ml. of medium was autoclaved, one of the necks being fitted with a potassium chloride bridge extension from a calomel electrode dipping into the liquid. On cooling, an extended glass electrode, sterilized in distilled water, was introduced into a second neck. The contents of the bottle were then inoculated and the electrodes connected to the recording pH meter. Samples were withdrawn as required through the third neck of the bottle.

A continuous record of mycelial weight could not be obtained from this apparatus; a set of 250-ml. flasks was therefore inoculated in parallel groups of four flasks chosen by lot removed from the shaker from time to time, and the bulked mycelial weight calculated to give that from 500 ml. of medium. Growth in the flasks was in general a little ahead of that in the bottle and Figures 3-8 must be interpreted with this fact in mind.

III. DETERMINATION OF ENZYMES AND OTHER COMPONENTS OF THE CULTURE MEDIUM

(*a*) C_x *Enzyme and Other* β*-Glucosidases*

The procedure for the C_x enzyme using sodium carboxymethyl cellulose as substrate has been outlined by Jermyn (1952*a*). A similar procedure with 1 per cent. solution of sodium cellulose sulphate (Eastman Kodak Co.) as substrate was also used.

The measurement of salicinase and *p*-nitrophenyl-β-glucosidase has also been outlined by Jermyn (1952*a*).

(*b*) *Amylase and Sucrase*

The procedure of Crewther and Lennox (1952) was used.

(*c*) *Proteases*

The activity of enzyme solutions in reducing the viscosity of gelatin was measured according to the method of Lennox and Ellis (1945). Their action on gelatin was also measured by the gravimetric technique of Crewther (1952). These are the "viscometric" and "gravimetric" enzymes of Crewther and Lennox (1953).

Table 1

GROWTH OF *S. ATRA* ON ACETATE MEDIA, CONTAINING THE SAME MINERAL ELEMENTS AS THE WAKSMAN-CAREY MEDIUM AND FURTHER SUPPLEMENTED BY THE ADDITION OF POSSIBLE MICRONUTRIENTS

Initial pH 7.0; cultured 5 days at 28°C.

Medium	Composition of Medium (g./l.)	Yield (mg./ml.) (mean of 6 flasks)
1	C, 8·0; N, 0·6; K, 14·8; P, 0·2; Na, 200×10^{-3}; Cl, 300×10^{-3}; S, 66×10^{-3}; Mg, 50×10^{-3}; Ca, 7×10^{-3}; Fe, 2×10^{-3}; Zn, 450×10^{-6}; Mn, 250×10^{-6}; biotin, 10×10^{-6}	1·44
2	As 1 plus Co, 20×10^{-6}; Cu, 12×10^{-6}; Mo, 25×10^{-6}	1·56
3	As 2 plus I, 30×10^{-6}; B, 3×10^{-6}	1·60
4	As 3 plus nicotinamide, 500×10^{-6}; thiamin hydrochloride, 200×10^{-6}; *p*-aminobenzoic acid, 100×10^{-6}; riboflavin, choline chloride, sodium pantothenate, pyridine hydrochloride; all 50×10^{-6}	1·79
5	As 4 plus peptone, 5×10^{-3}	1·86
6	As 5 plus F, 45×10^{-6}; Br, 35×10^{-6}; Cd, 20×10^{-6}; Ni, 12×10^{-6}; Al, 4×10^{-6}	2·02
7	As 6 plus Hg, 40×10^{-6}; Ba, 25×10^{-6}; Cr, 10×10^{-6}	1·90

(*d*) *Esterase*

This enzyme was measured by its ability to decompose the diacetyl derivative of chlorphenol red (3×10^{-5}M) at pH 7.0 and 37°C.

(*e*) *Reducing Sugar*

The reducing sugar in the suitably diluted culture filtrate was estimated by the Nelson-Somogyi colorimetric method (Somogyi 1945). All substances present capable of reducing alkaline copper reagents would be estimated as "reducing sugar" by this technique. Since the apparent reducing sugar in the medium usually fell to zero, error from this source could not have been very great.

(*f*) *Sucrose*

Sucrose was determined as in (*e*) after inversion with dilute hydrochloric acid.

TABLE 2

GROWTH OF *S. ATRA* IN A BASAL MEDIUM CONTAINING ACETIC ACID A.R. 15 ML., KNO_3 A.R. 1.0 G., $MgSO_4.7H_2O$ A.R. 0.5 G., KH_2PO_4 A.R. 3.0 G., KOH A.R. 16.5 G., AND D-BIOTIN 10 μG/L.

Medium adjusted to pH 7.0 (KOH) and trace elements removed according to the procedure of Steinberg (1950).

Additions						Yield of Mycelium (mg./ml.) After 5 Days at 28°C.
Fe^{++} (μg./l.)	Zn^{++} (μg./l.)	Mn^{++} (μg./l.)	Cu^{++} (μg./l.)	Mo (μg./l.)	Other Additions	
—	—	—	—	—	—	0·37
—	400	200	100	40	—	0·32
600	—	200	100	40	—	0·34
600	400	—	100	40	—	0·38
600	400	200	—	40	—	0·54
600	400	200	100	—	—	0·96
600	400	200	100	40	—	1·14
1200	400	200	100	40	—	1·30
600	800	200	100	40	—	2·28
600	400	400	100	40	—	1.66
600	400	200	200	40	—	1·26
600	400	200	100	80	—	0·60
600	400	200	100	40	I′ 300 μg./l., Br′ 250 μg./l., B 25 μg./l.	0·60
600	400	200	100	40	Plant ash (from wood of *Eucalyptus* sp.) 1 mg./l.	0·42
600	400	200	100	40	Mixed B vitamins at 10-100 μg./l.	1·96
600	400	200	100	40	Peptone 10 mg./l.	2·40
600	400	200	100	40	All the above additions	1·42

(*g*) *Total Carbohydrates*

The orcinol method of Pirie (1936) was suitable for the estimation of starch and its degradation products in S. *atra* culture filtrates.

(*h*) *Mycelial Weight*

The culture medium was filtered through a pair of matched ashless filter papers (Whatman No. 541), the mycelium washed once with distilled water, the whole dried 48 hr. at 55°C., and the mycelial weight obtained by difference.

IV. MINERAL NUTRITION OF S. ATRA

The modified Waksman-Carey solution of Fahraeus (1947) is deficient in certain elements known to be important in the nutrition of fungi, including zinc, copper, and molybdenum (Steinberg 1950). When S. *atra* was grown in this medium with glucose or sucrose as a carbon source, addition of other elements did not affect the final yield of mycelium. The addition of $ZnSO_4.7H_2O$ (2 mg./l.) was found to eliminate the lag periods of many days between the germination of the spores and the onset of rapid growth, which occurred in an unpredictable way in the absence of zinc. Apart from this regulatory effect, ordinary "purified" sugars and A.R. chemicals apparently contained enough of the micronutrient elements for growth of the fungi.

TABLE 3

COMPARISON OF MINERAL NUTRITION OF *S. ATRA* WITH THAT OF *M. ECHINATA* (PERLMAN 1948)

Metal	S. *atra*, Present Work, Acetate Media		*M. echinata*, Perlman (1948), Glucose Media		
	Optimum Concentration (μg./l.)	Ratio of Mycelial Yield at Optimum to Mycelial Yield on Basic Medium	Optimum Concentration (μg./l.)	Concentration in Basic Medium (μg./l.)	Rate of Mycelial Yield at Optimum to Mycelial Yield on Basic Medium
Iron	600-1200	*c.*3	230-430	136	1·9
Zinc	>800	At least 6·5	*c.* 110	11	1·9
Manganese	>400	At least 4	*c.* 1000	3	2·3
Copper	100-200	*c.* 2·2	*c.* 300	1	1·3

When S. *atra* was grown on acetate media its dependence on various inorganic micronutrients became apparent. It was possible to show that the presence of all the mineral components of the Waksman-Carey medium except calcium was necessary for optimum growth when using unpurified reagents. Table 1 shows that certain further additions to the medium brought about stimulation of growth under the conditions used by a further 40 per cent. These observations were amplified by testing certain micronutrients in the more rigorous experiments set out in Table 2. Specially cleaned, silicone-treated glassware was used, the basal medium was purified by the calcium carbonate method of Steinberg (1950) and washed-spore suspensions were used for inoculation. Significant but not absolute dependence on iron, manganese, copper, and zinc was demonstrated, together with an apparently inhibitory effect of molybdenum in excess of 40 μg./l. Fair growth of the mould in "purified" basal medium apparently means that Steinberg's procedure is not completely effective in removing trace metals from acetate media. In Table 3 the results for the growth of S. *atra* are compared with those of Perlman (1948) for the growth of *M. echinata* in glucose media.

TABLE 4

UTILIZATION OF VARIOUS CARBON SOURCES BY *S. ATRA* GROWING ON A WAKSMAN-CAREY MINERAL MEDIUM

Concentration of carbon source, 1 per cent.; initial pH 7.0; grown 5 days at 28°C.

Carbon Source	Yield (mg./ml.)	Carbon Source	Yield (mg./ml.)
(*a*) Sugars		Cellulose: filter paper	++++§
D-Fructose	7·7	sawdust	+++§
Maltose	4·2	Sodium alginate	Nil
D-Galactose	4·1	Cellulose acetate	Nil
D-Glucose	3·2	(*e*) Miscellaneous sugar	
D-Mannose	2·1	derivatives	
D-Xylose	3·0	Gluconic acid	3·0‡
L-Rhamnose	2·9	Galactonic acid	Nil
Lactose	2·7	Gulonic acid	Nil
Sucrose	2·5	Ribonic acid	Nil
Cellobiose	2·3	Saccharic acid	Nil
Trehalose	1·9	Mucic acid	Nil
Raffinose	1·8	Fructose diphosphate	Nil
D-Lyxose	0·9	Cellobiose octa-acetate	+§
D-Arabinose	0·7	(*f*) Alcohols	
D-Ribose	0·3	Methanol	Nil
L-Arabinose	0·8*	Ethanol	Nil
D-Fucose	Nil	*iso*-Propanol	Nil
L-Xylose	Nil	*iso*-Butanol	Nil
L-Sorbose	Nil	*n*-Butanol	Nil
D-Glucoseamine	Nil	*tert*-Pentanol	Nil
(*b*) Sugar alcohols		*n*-Pentanol	Nil
Glycerol	5·3	*n*-Hexanol	Nil
Mannitol	3·2	Ethylene glycol	Nil
Arabitol	1·0	Propylene glycol	Nil
Dulcitol	0·9	(*g*) Organic acids	
Adonitol	0·8†	Acetate	0·7
Inositol	0·3†	Propionate	+¶
Sorbitol	Nil	Butyrate	+
Erythritol	Nil	*iso*-Butyrate	+
(*c*) Glucosides		Valerate	+
β-Methyl-glucoside	1·9	Oxalate	+
α-Methyl-glucoside	1·3	Malonate	+
Salicin	1·3	Succinate	+
(*d*) Polysaccharides		Adipate	+
Inulin	3·6	Malate	0·1
Sodium carboxymethyl		Lactate	+
cellulose	2·5	Citrate	+
Starch	2·1	Benzoate	+
Dextrin	1·7	Salicylate	+
Pectin	2·5‡	Formate	Nil
Arabinic acid	1·3‡	Glycollate	Nil
Xylan	++++§	Tartrate	Nil
Chitin	++++§	Glycerophosphate	Nil
Cellulose: cotton wool	++++§	Pyruvate	Nil

* After 13 days incubation.

† After 10 days incubation.

‡ After 7 days incubation.

§ Residual insoluble substrate prevented mycelial weight determination.

¶ "+" indicates growth in non-weighable amounts.

TABLE 4 (*Continued*)

Carbon Source	Yield (mg./ml.)
(*h*) Organic acids; nitrate as N source	
Acetate	1·0
Malonate	0·5
Malate	0·4
Tartrate	0·1
Succinate	0·1
iso-Butyrate	+
Butyrate	+
Lactate	+
Citrate	+
Oxalate	+
Formate	Nil
Valerate	Nil

Mixed B vitamins and protein nitrogen exert an apparent stimulatory effect on the growth of S. *atra*; in view of the results of Marsh and Bollenbacher (1946) and Perlman (1948) this is unlikely to reflect the existence of absolute requirements.

V. UTILIZATION OF CARBON AND NITROGEN SOURCES

(*a*) *Carbon Sources*

The substance to be tested was added to the basal medium at a final concentration of 1 per cent., and where necessary the pH of the medium was readjusted to 7.0. The results of tests using a number of substrates are set out in Table 4.

(*b*) *Nitrogen Sources*

The carbon source used was 1 per cent. glucose and a few experiments with acetate media are also reported. The medium was varied by adding K_2HPO_4 (3.3 g./l.) in place of $(NH_4)_2HPO_4$ and a nitrogen source equivalent to pH 7.0 The results are set out in Table 5.

(*c*) *Single Substrates as both Carbon and Nitrogen Sources*

A certain number of the amino acids and proteins were tested as sources of both carbon and nitrogen. The $(NH_4)_2HPO_4$ in the medium was again replaced by K_2HPO_4 and the nutrient was added in an amount sufficient to give 0.57 g. N/l. These results are set out in Table 6.

(*d*) *Adaptation to Various Media*

The results quoted in Tables 4, 5, and 6 are for direct transfer from growth on the potato-dextrose agar solid medium to growth in liquid culture. All media on which fair growth was not apparent by the fifth day were retained

Table 5

UTILIZATION OF VARIOUS NITROGEN SOURCES BY *S. ATRA* GROWING ON A WAKSMAN-CAREY MINERAL MEDIUM

Carbon source: 1 per cent. glucose; initial pH 7.0; incubated 5 days at 28°C.

Nitrogen Source	Yield (mg./ml.)
(*a*) Inorganic	
Potassium nitrate	6·4
Potassium nitrite	4·0
Ammonium phosphate	3·2
Ammonium nitrate	2·9
Ammonium sulphate	2·7
Ammonium chloride	2·1
Ammonium carbonate	0·9
Potassium thiocyanate	+
Potassium cyanide	Nil
Hydroxylamine hydrochloride	Nil
Hydrazine sulphate	Nil
(*b*) Amines	
Methylamine	Nil
Dimethylamine	1·5
Trimethylamine	1·2
Tetramethylammonium bromide	+[*]
Ethylamine	1·7
n-Propylamine	0·4
iso-Butylamine	0·3
n-Amylamine	Nil
Ethanolamine	0·9
Ethylenediamine	Nil
Putrescine	0·2
Cadaverine	+
Pyridine	+
Piperidine	+
α-Amino pyrimidine	+
Indole	Nil
(*c*) Urea and related substances	
Methyl urea	5·1
Ethyl urea	4·9
Guanidine hydrochloride	4·5
Alloxan	4·2
Allantoin	3·7
Urea	3·2
Methylisothiourea sulphate	1·7
Nitroguanidine	1·2
Phenylurea	1·1
Creatine	0·8
Creatinine	0·5
Thiourea	+
(*d*) Amides	
Acetamide	0·9
Potassium phthalimide	3·1
Urethane	+
Hippuric acid	Nil
(*e*) Purines	
Xanthine	4·0
Uric acid	3·1
Caffeine	+
Theobromine	+
(*f*) Growth factors	
Choline chloride	2·3
Nicotinamide	0·3
Pyridoxine hydrochloride	0·3
p-Aminobenzoic acid	Nil
Thiamine chloride	Nil
(*g*) Amino acids	
Proline	5·7
Arginine	5·5
Glycine	4·9
Valine	4·1
Asparagine	3·4
Phenylalanine	3·1
Leucine	2·2
Lysine	1·9
Isoleucine	1·8
Histidine	1·5
Cystine	++++[†]
Hydroxyproline	+
Aspartic acid	Nil
Glutamic acid	Nil
(*h*) Proteins	
Gelatin	3·3
Peptone	2·6
(*i*) Various N sources with acetate media	
Ammonium nitrate	4·6
Potassium nitrate	1·0
Ammonium phosphate	0·7
Urea	0·5
Arginine	0·45
Guanidine	0·4
Proline	0·35
Methylurea	0·35
Uric acid	0·3
Glycine+potassium nitrate	0·3
Glycine	0·2
Potassium nitrite	0·2

[*] "+" Indicates growth in non-weighable amounts.

[†] Residual soluble substrate prevented mycelial weight determination.

on the shaking machine for 3 weeks; if no growth was apparent after this period the cultures were discarded. In a few cases, noted in the tables, scanty growth took place in this period.

TABLE 6

UTILIZATION OF VARIOUS SUBSTRATES AS CARBON AND NITROGEN SOURCES BY *S. ATRA* GROWING ON WAKSMAN-CAREY MEDIUM

Initial pH 7.0, incubated 5 days at 28°C.

Subtrate	Yield (mg./ml.)
(*a*) Amino acids	
Alanine	0·3
Valine	0·15
Proline	0·04
Arginine	0·03
Histidine	0·03
Glycine	+*
Lysine	+
Isoleucine	+
Asparagine	+
Phenylalanine . .	+
Cystine	Nil
Leucine	+
Hydroxyproline . .	+
(*b*) Proteins	
Haemoglobin . .	3·0
Gelatin	2·8
Tryptone	2·1
Zein	2·0
Egg albumen	1·9
Peptone	1·9
"Mould enzyme" . .	1·2
Casein	0·5
Ground wool	++++†

* "+" Indicates growth in non-weighable amounts.

† Residual insoluble matter prevented mycelial weight determination.

Mycelial transfers were made from sparse cultures to a fresh batch of medium and this process was continued so long as there were signs of adaptation to the new substrate. Serial transfers were also made from cultures on various media that had supported measurable amounts of growth in the original experiments. The results of these subcultures may be summarized as follows:

(i) Media supporting "good" growth (better than about 0.5 mg./ml. in 5 days) continued to support growth at least as well on continued subculture.

(ii) Some media that gave scanty growth ("+") supported luxuriant growth on continued subculture; in other cases the amount of visible growth decreased to zero after two or three subcultures. Thus adaptation was made to growth on media with propionate and malate as carbon sources and on cyanide as a nitrogen source, but subculture proved impossible on media with succinate and lactate as carbon sources. It was eventually possible to transfer to the corresponding solid medium *S. atra* that had become adapted to growth in liquid media; the fungus has, for instance, been maintained for over a year on propionate — Waksman-Carey agar. The range of nitrogen sources that could be utilized by these adapted strains was much diminished, nitrate being strongly preferred.

(iii) The adaptation of *S. atra* to growth on media containing salicylate and benzoate was accompanied by visible "mutation" and after about 20 days of culture on the salicylate medium, *S. atra* began to show sparse growth. Subculture on salicylate medium led to profuse growth with the production of large quantities of insoluble yellow pigment. Transfer of the culture to agar slopes, whether of solidified salicylate medium or potato dextrose agar, gave olive colonies that showed no sign of reversion to the wild type.

Microscopically the cultures were characterized by globose spores borne on exceptionally long conidiophores. White, Yeager, and Shotts (1949) have shown that aggregates of *Stachybotrys* spores always contain a few globose spores of the *Memnoniella* type; their observations have been confirmed in this laboratory. When spores from successive subcultures on liquid salicylate media were examined, it was found that replacement of the ellipsoid *Stachybotrys* type by the globose *Memnoniella* type occurred over three transfers. Intermediate cultures showed both spore forms.

It would seem that *S. atra* is heterokaryotic, with salicylate acting as a selecting agent.*

VI. Growth and Enzyme Production on Various Media

(*a*) *General Observations*

The composition of the modified Waksman-Carey medium is such that growth of a micoorganism upon it with the consequent depletion of the ammonium nitrogen must lead to a sharp fall in pH. With *S. atra* the onset of growth is sudden and the consequent fall in pH extremely sharp; with no experimental technique so far devised has it been possible to synchronize this change in a large number of flasks.

The occurrence of the second smaller drop in pH during growth on sugar media is particularly erratic and no account of the factors controlling its appearance can yet be given. Discrepancies between the "chronological" and "physiological" ages of cultures vitiated all experiments in which flasks were removed from the shaker at intervals and the culture filtrates examined. The Woulffe-bottle method for following a single culture was therefore devised to overcome this difficulty.

It might appear that these troubles could be eliminated by buffering the medium; in Table 7 are set out the results of a series of experiments in which media were prepared containing increasing proportions of 1.0M $K_xH_{3-x}PO_4$, pH 7.0. The table shows that reproducible growth is not obtained even when the pH of the medium is held relatively steady.

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* Morphological details of the new strain and a full account of the above and further experiments will be published elsewhere.

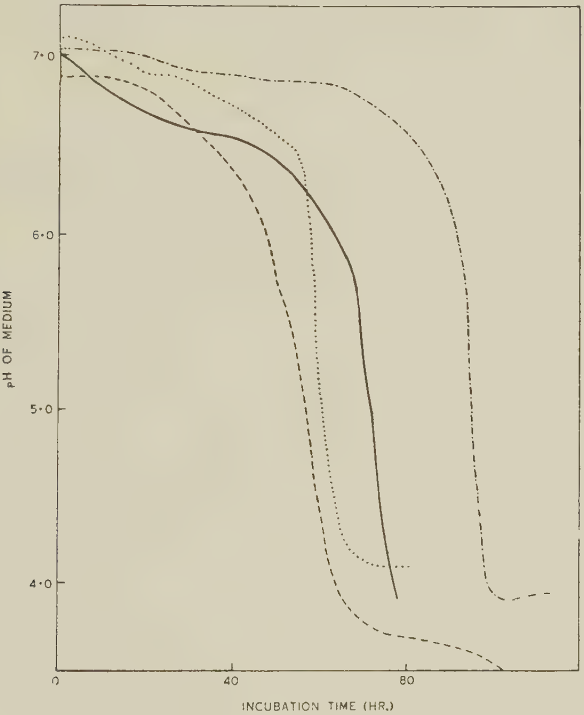

Fig. 1.—Changes in pH during the first 100 hr. of growth of S. *atra* on Waksman-Carey medium containing 2 per cent. of glucose. Four separate Woulffe-bottle cultures at 28°C.; pH followed on the recording pH meter.

The causes of the inherent variability in growth cannot at present be defined; in the experiments on the nutrition of the mould small amounts of vitamins or proteins added to the medium gave cultures with much reduced variability (cf. Table 1). It is possible that random variation in the carry-over of such stimulatory substances in the inoculum was responsible for the observed results.

Examination of culture filtrates by solvent extraction after the addition of excess mineral acid, followed by chromatographic investigation of the extract, showed the absence at all stages of organic acids in other than trace amounts. This is in agreement with the observations of Perlman (1948) on *M. echinata.* The production of organic acids by S. *atra* thus plays no part in the observed pH changes of the medium.

(*b*) *Changes in pH During Growth*

The variability of the time-pH curve for the first 100 hr. of growth on a medium containing 2 per cent. glucose is illustrated in Figure 1 for a number

TABLE 7

GROWTH OF *S. ATRA* ON WAKSMAN-CAREY MEDIUM CONTAINING VARIOUS CARBON SOURCES AND DILUTED WITH VARYING AMOUNTS OF 1.0M PHOSPHATE BUFFER OF pH 7.0

Individual 250-ml. flasks in shake culture at 28°C., containing 50 ml. of medium. "Enzyme activity" is the hydrolytic activity against sodium carboxymethyl cellulose

Volume per cent. of Phosphate Buffer in Final Medium	Carbon Source								Days Incubated
	Glucose (2%)			Sucrose (2%)			Cotton Wool (60 mesh: 2%)		
	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	
0	3·60	1·46	103	2·20	0·49	231	6·58	0·11	3
	3·42	0·77	94	3·40	1·08	88	5·90	0·08	4
	3·48	0·83	90	3·55	0·99	94	5·60	0·28	5
	3·40	0·88	100	3·58	0·62	105	5·92	0·31	6
	3·40	0·50	108	3·32	0·88	101	5·45	0·18	7
	3·38	0·87	98	3·30	1·00	115	5·30	0·28	10
	3·10	0·83	102	3·26	1·31	100	5·75	0·27	12
	3·42	1·28	100	2·20	0·12	379	5·12	0·23	14
5	3·58	0·94	36	6·38	1·33	60	6·80	0·10	3
	3·70	0·29	135	4·30	0·51	244	6·75	0·05	4
	3·60	0·60	110	4·05	0·22	180	6·68	0·21	5
	3·85	0·62	61	6·12	0·09	173	6·62	0·24	6
	3·62	0·52	63	5·92	0·03	236	6·52	0·27	7
	6·25	0·32	263	6·00	0·13	262	6·42	0·30	10
	3·78	0·53	100	6·30	0·04	225	6·50	0·40	12
	3·80	0·35	130	6·35	0·20	179	6·45	0·53	14
10	6·00	1·05	114	6·55	1·22	51	6·90	0·14	3
	6·15	0·10	178	6·18	0·22	241	6·82	0·10	4
	6·05	0·27	161	5·82	0·03	258	6·82	0·32	5
	6·20	0·29	158	6·28	0·13	187	6·70	6·35	6
	6·30	0·53		6·30	0·10	187	6·60	0·58	7
	6·50	0·14	130	6·15	0·02	215	6·70	0·70	10
	6·48	0·37	187	6·48	0·18	186	6·70	1·07	12
	6·52		110	6·28	0·02	264	6·50	0·69	14
20	6·70	1·34	48	6·50	0·93	48	6·92	Nil	3
	6·52	0·18	184	6·10	0·13	204	6·98	Nil	4
	6·50	0·04	203	6·40	0·32	171	6·98	0·02	5
	6·75	0·40	186	6·52	0·25	187	6·52	0·35	6
	6·52	0·10	133	6·65	0·13	163	6·80	0·52	7
	6·65	0·22	55	6·60	0·17	161	6·52	0·43	10
	6·80	0·38	299	6·80	0·08	176	6·52		12
	6·62	0·37	237	6·68	0·26	70	6·68	0·83	14

TABLE 7 (*Continued*)

Volume per cent. of Phosphate Buffer in Final Medium	Carbon Source								Days Incubated
	Glucose (2%)			Sucrose (2%)			Cotton Wool (60 mesh: 2%)		
	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	Mycelial Weight (mg.)	pH of Medium	Enzyme Activity	
35	6·80	1·22	30	6·92	0·50	19	7·00	0·10	3
	6·62	0·33	148	6·80	1·15	57	6·98	0·07	4
	6·50	0·10	108	6·70	1·53	35	6·95	0·28	5
	6·65	0·35	150	6·60	0·32	116	6·95	Nil	6
	6·50	0·34	124	6·62	Nil	221	6·80	0·14	7
	6·70	0·03	151	6·75	0·88	153	6·62	1·13	10
	6·80	0·04	87	6·92	0·06	251	6·90	0·17	12
	6·72	0·64	194	6·75	0·05	173	6·75	0·70	14
50	6·90	1·20	6	6·85	0·32	11	6·88	0·47	3
	6·88	1·35	10	6·95	1·43	21	7·00	0·25	4
	6·80	1·18	30	6·50	0·78	8	6·88	Nil	5
	6·55	0·24	141	6·75	0·37	30	6·90	Nil	6
	6·52	0·19	162	6·70	Nil	47	6·70	Nil	7
	6·52	0·04	115	6·72	0·14	5	6·82	1·06	10
	6·85	0·27	23	6·75	0·78	25	6·90	Nil	12
	6·70	0·28	165	6·70	0·09	40	6·75	0·27	14

of experiments. Figure 2 illustrates the complete course of the time-pH curves during typical growth on media containing 1, 3, and 5 per cent. glucose.

(*c*) *Enzyme Production on Various Media*

In Figures 3 and 4 are set out the essential details of growth on unmodified Waksman-Carey media with 2 per cent. glucose and sucrose, respectively, as the carbon sources. There is an overall similarity in the relation between C_x activity, mycelial weight, pH of medium, and utilization of sugar for cultures on the two media. Figure 4 shows the tendency, which has been general in these experiments, for salicinase to follow the same course as C_x and the *p*-nitrophenyl-*β*-glucosidase to pursue an independent course.

The data on which Figure 3 is based were secured before the importance of checking the activity of a number of enzymes was realized and no further continuous runs on this simple unmodified medium have been made. Later independent experiments have shown that the proteases (gravimetric and viscometric) are not demonstrable at any stage of growth; sporadic and extremely small activities can be demonstrated for esterase, amylase, and sucrase between the fifth and tenth days of growth.

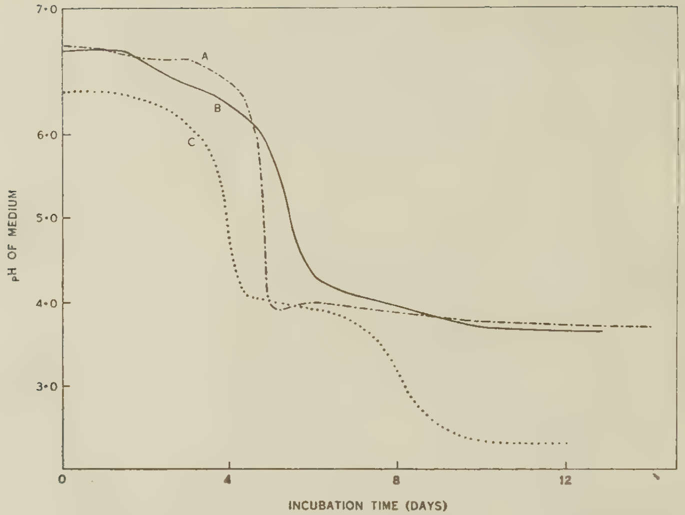

Fig. 2.—Changes in pH during the growth of S. *atra* on Waksman-Carey medium plus glucose. Woulffe-bottle cultures at 28°C.; pH followed on the recording pH meter. *A*, 1 per cent. glucose; *B*, 3 per cent. glucose; *C*, 5 per cent. glucose.

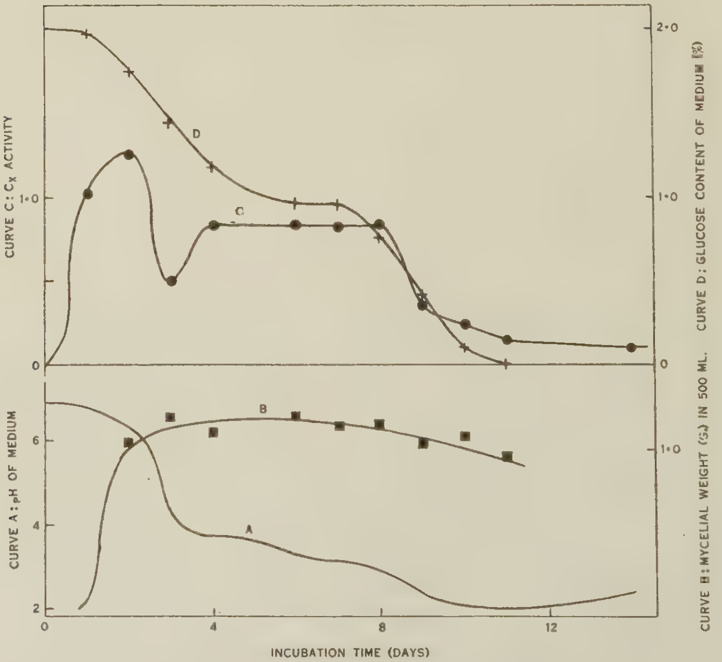

Fig. 3.—Growth of S. *atra* on 2 per cent. glucose medium. *A*, pH of medium; *B*, mycelial weight; *C*, "C_x" activity; *D*, sugar content of medium.

The growth of *S. atra* was also followed on glucose media modified in various ways; the results are set out in Figures 5, 6, and 7. It is apparent that each modification induces its own particular variation in the pattern of growth and enzyme production. In no case could the formation of significant amounts of amylase, sucrase, esterase, or protease be detected.

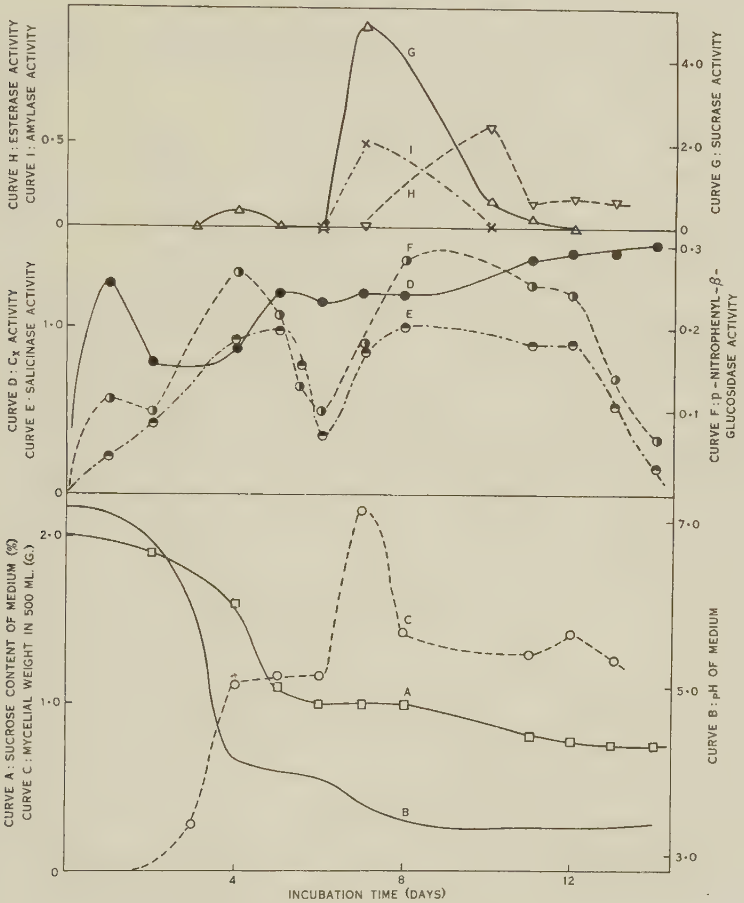

Fig. 4.—Growth of *S. atra* on 2 per cent. sucrose medium. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity; *G*, sucrase activity; *H*, esterase activity; *I*, amylase activity.

When glucose is replaced by starch (Fig. 8), esterase, amylase, and sucrase are produced in some quantity and there is a general tendency for periods of maximum and minimum activity of these enzymes in the culture filtrate to coincide with those of the enzymes splitting β-glucosidic linkages. Of these latter enzymes C_x and salicinase do not exceed the maximum activities found in sugar media, but there is great increase in the *p*-nitrophenyl-β-glucosidase.

(*d*) *Comparison of Certain β-Glucosidase Activities in the Culture Media*

The comparative hydrolytic activity against salicin, *p*-nitrophenyl-β-glucoside, sodium carboxymethyl cellulose, and sodium cellulose sulphate was determined in bulked culture filtrates with the extra accuracy allowed by large sample size. The results are set out in Table 8.

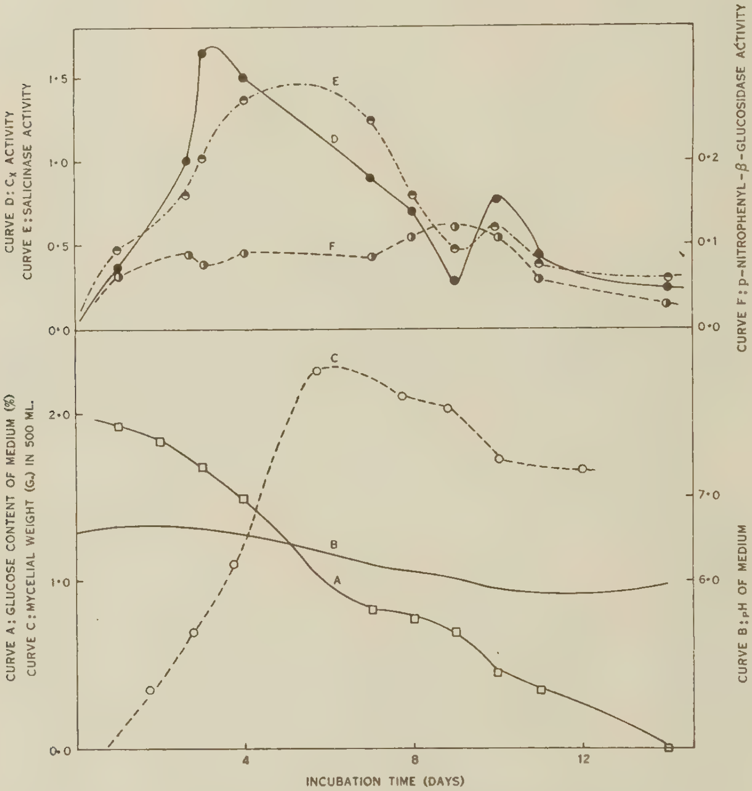

Fig. 5.—Growth of S. *atra* on 2 per cent. glucose medium plus 1/5 vol. of 1.0M phosphate buffer of pH 7.0. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

VII. Discussion

There is no evidence of any unusual physiological characteristics in S. *atra*. The lists of available nitrogen and carbon sources supplement those of Perlman (1948) without any essential disagreement. The mould is not restricted to any type of nitrogen source, but as carbon sources shows a marked preference for sugars, polysaccharides, glucosides, and sugar alcohols, simpler molecules being utilized only in the presence of nitrate nitrogen.

TABLE 8

COMPARISON OF VARIOUS β-GLUCOSIDASE ACTIVITIES IN BULKED CULTURE FILTRATES

Carbon Source	Days of Growth	Hydrolytic Activity Against						
		p-Nitrophenyl-β-glucoside		Salicin		Sodium Cellulose Sulphate		Sodium Carboxymethyl Cellulose
		Arbitrary Units	SCMC-ase=1	Arbitrary Units	SCMC-ase=1	Arbitrary Units	SCMC-ase=1	Arbitrary Units
Glucose	2	9·0	0·11	64	0·75	86	1·0	85
	7	9·0	0·36	18	0·7	27	1·1	25
Sucrose	2	16·0	0·34	44	0·95	72	1·5	47
	5	8·7	0·14	37	0·65	47	0·75	64

Growth on unbuffered sugar media shows a definite sequence of phases:

(*a*) Germination, with considerable liberation of β-glucosidases before there is much apparent mycelial growth.

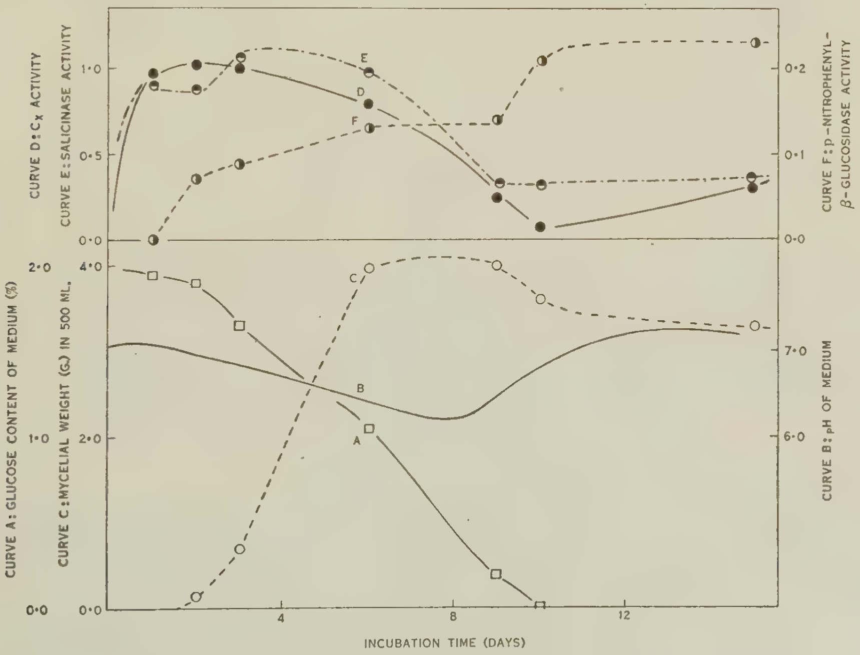

Fig. 6.—Growth of *S. atra* on 2 per cent. glucose medium plus 1 per cent. (w/v) of $CaCO_3$. *A*, sugar content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

(*b*) Rapid growth, with fall in pH, consumption of sugars, and loss of C_x and salicinase activity.

(*c*) A steady period at pH 3-4, with a new rise in C_x and salicinase activity and little or no utilization of sugar.

(*d*) Renewed utilization of sugar, with a further slight decrease in pH and loss of C_x and salicinase activity.

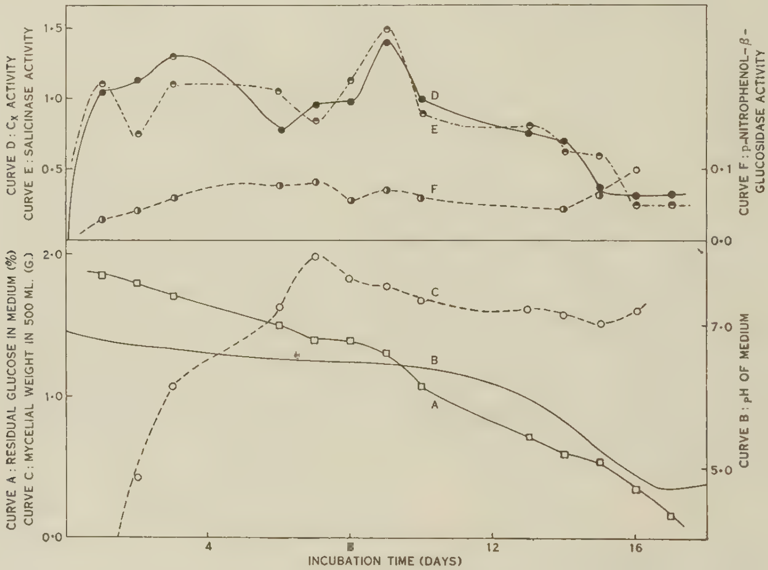

Fig. 7.—Growth of S. *atra* on 2 per cent. glucose medium modified by replacing $(NH_4)_2HPO_4$ with $K_2HPO_4 + KNO_3$. *A*, sugar content of medium; *B*, pH; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity.

It is noteworthy that the first of these phases is not much altered by the addition of buffering agents or the replacement of ammonium by nitrate; secretion of relatively large amounts of β-glucosidases at, or just after, germination appears to be a constant feature of the metabolism of S. *atra*. Since the "C_x" activity is part of "cellulase" activity this early secretion of enzyme will be of the utmost importance for the mould in gaining a foothold on cellulosic substrates, and may be decisive in securing for S. *atra* the dominant position in the cellulolytic flora which it so often occupies in the field.

The secretion of "C_x" and salicinase appears to occur in bursts, with slow inactivation of the secreted enzyme thereafter; this is shown most clearly by the data for the two buffered glucose media, where the highest peak of activity occurs early in the history of the culture, and is followed by a nearly constant decline. On the limited evidence of the starch medium, this conclusion appears to be true for other enzymes also. The factor limiting growth in the media used was nitrogen; calculations show that approximately enough is present to synthesize about 1.6 g. of protein per 500 ml. of medium. Inspection of the figures shows that except for the medium containing $CaCO_3$ growth ceased at a figure of about 2 g. of mycelium per 500 ml., followed by a drop in mycelial weight, and the liberation of enzymes. There were marked changes in pH, and the sugar continued to be utilized (presumably by respiration to CO_2). This partial autolysis could not be pushed further, however, and incubation for indefinite periods showed an almost constant mycelial weight. The residual mycelium was autolysed in all experiments (18 hr. with ethyl ether at 1°C.) but significant amounts of saccharases were not recovered.

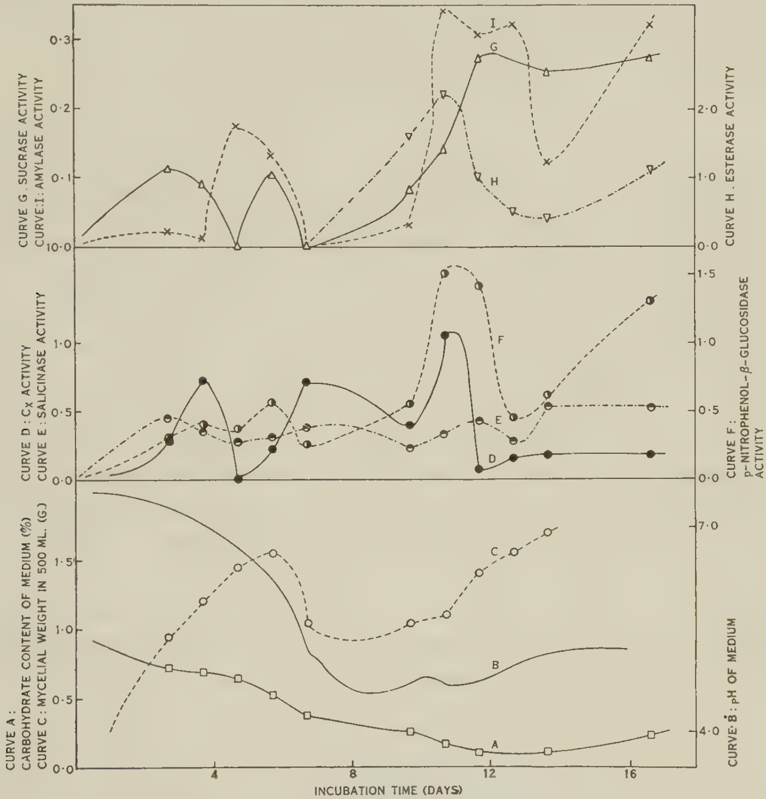

Fig. 8.—Growth of *S. atra* on 1 per cent. starch medium. *A*, carbohydrate content of medium; *B*, pH of medium; *C*, mycelial weight; *D*, C_x activity; *E*, salicinase activity; *F*, *p*-nitrophenyl-β-glucosidase activity; *G*, sucrase activity; *H*, esterase activity; *I*, amylase activity.

Shu and Blackwood (1951) have studied the production of certain carbohydrases by *A. niger* growing in submerged culture. Alpha-amylase and maltase activity were strongly dependent on the carbon source used, being higher on starch, dextrin, or maltose than on carbon sources not containing α-glucosidic linkages; "limit-dextrinase" activity on the other hand was almost independent of the carbon source. This situation is very similar to that found in this study for *S. atra*, amylase and sucrase production being dependent on the carbon source, and that of the C_x enzyme and other β-glucosidases being relatively independent. Jermyn (1952*a*) has already shown that in general somewhat higher levels of the C_x enzyme are produced on media containing carbon sources with β-glucosidic linkages, so that this enzyme might be classified as "adaptive" if a few results only were considered. Contrary to the conclusions of Reese, Siu, and Levinson (1949) for cellulolytic moulds in general, however, its formation in large quantities by *S. atra* is possible in the absence of cellulose derivatives as carbon sources. The results obtained are apparently dependent on the balance of a number of factors, and this type of situation is fairly general; Gershenfeld and Wasserman (1950) found that, by choosing certain initial conditions, it was possible to demonstrate an apparently higher production of sucrase by *Neurospora crassa* growing on xylose, raffinose, and fructose than on sucrose, although a more complete study showed this enzyme to be partially adaptive.

The salicinase and C_x enzyme activities of *S. atra* follow courses that are sufficiently close to make it credible that the same enzymes are involved, although inspection of Figures 3-8 and of Table 8 shows that this relationship is not absolute; *p*-nitrophenyl-β-glucosidase appears to bear little or no relationship to the other two activities. The explanation advanced by Jermyn (1952*b*) for the properties of the *A. oryzae* β-glucosidases may therefore be applicable to those of *S. atra* and we may here have another example of a group of β-glucosidases each with its own ratio of activities towards different substrates, the sum of these activities being measured as the apparent activities of such "enzymes" as "salicinase" and "C_x." This hypothesis receives support from the tendency of salicinase and the C_x enzyme to have parallel maxima and minima of activity that are a day or two apart, as if a certain set of conditions was operating to produce or inactivate the β-glucosidases, but individual enzymes responded slightly differently.

The present study therefore gives no support for the existence of "C_x" as separate enzyme, hydrolysing polymeric β-glucosidic linkages only, and produced by the mould solely in response to the presence of these linkages.

VIII. ACKNOWLEDGMENTS

The author is deeply indebted to Miss B. Lyons and Mrs. M. C. Wilkinson for their able technical assistance.

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TRANSFERASE ACTIVITY OF THE β-GLUCOSIDASES OF *ASPERGILLUS ORYZAE*

By M. A. Jermyn* and R. Thomas*

[*Manuscript received October* 2, 1952]

Summary

The *p*-nitrophenyl-β-glucosidase of *Aspergillus oryzae* is able to transfer a glucose residue to methanol, ethanol, and *n*-butanol. The enzymes hydrolysing salicin and cellobiose carry out this transfer to a much less degree if at all. There is evidence that these enzymes can transfer glucose residues to other sugar molecules to build up more complex saccharides.

I. Introduction

Takano and Miwa (1950) have shown that there exist in the apricot (*Prunus armenica*), *Aspergillus niger*, and *Penicillium chrysogenum* enzymes capable of transferring the β-glucosyl residue of *p*-nitrophenyl-β-glucoside to methanol and butanol to give the corresponding glucosides. They concluded from observation of the parallel changes in the two activities during purification that the glucotransferase and β-glucosidase were identical in all three cases.

Pazur and French (1951) found that culture filtrates of *A. oryzae* contained a transglucosidase catalysing such reactions as:

$$\underset{\text{(maltose)}}{4(\alpha\text{-D-glucopyranosyl})\text{-D-glucose}} + \underset{\text{(isomaltose)}}{6(\alpha\text{-D-glucopyranosyl})\text{-D-glucose}}$$
$$\rightarrow \text{glucose} + \underset{\text{(dextrantriose)}}{6(\alpha\text{-isomaltosyl})\text{-D-glucose}}$$

In this case an enzyme with α-glucosidase activity acts as a transferase to build up a dextran chain by transglucosidation. Indeed it seems that none of the carbohydrases act only as simple hydrolases. Wallenfels (1951) observed formation of galactosidolactose from lactose in the presence of *A. oryzae* extracts. Bacon and Edelman (1950) have shown that even in the classic case of invertase, hydrolysis is accompanied by transglycosidation and the synthesis of oligosaccharides.

The present study arose from observation of the non-equivalence of the glucose and *p*-nitrophenol produced during the hydrolysis of *p*-nitrophenyl-β-glucoside by enzymes of *A. oryzae* (Jermyn 1952*a*). All fissions of β-glucosidic linkages by these enzymes have kinetics too complex to be those of simple hydrolyses.

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II. Experimental

Enzymic hydrolysis of *p*-nitrophenyl-β-glucoside was followed by determining the glucose and *p*-nitrophenol produced at 37°C. in a mixture of 50 ml. of 10^{-3}M glucoside, 20 ml. of pH 5.0 McIlvaine citrate-phosphate buffer, enzyme solution, alcohol, and water to give a total volume of 100 ml. Samples were heated in boiling water for 5 min., glucose determined by the Somogyi-Nelson micromethod (Somogyi 1945) after correction for reduction by the enzyme itself, and *p*-nitrophenol by direct colorimetry. Neither *p*-nitrophenol nor its glucoside had a reducing effect on the Somogyi-Nelson reagents, nor did they affect the reduction of the reagent by glucose.

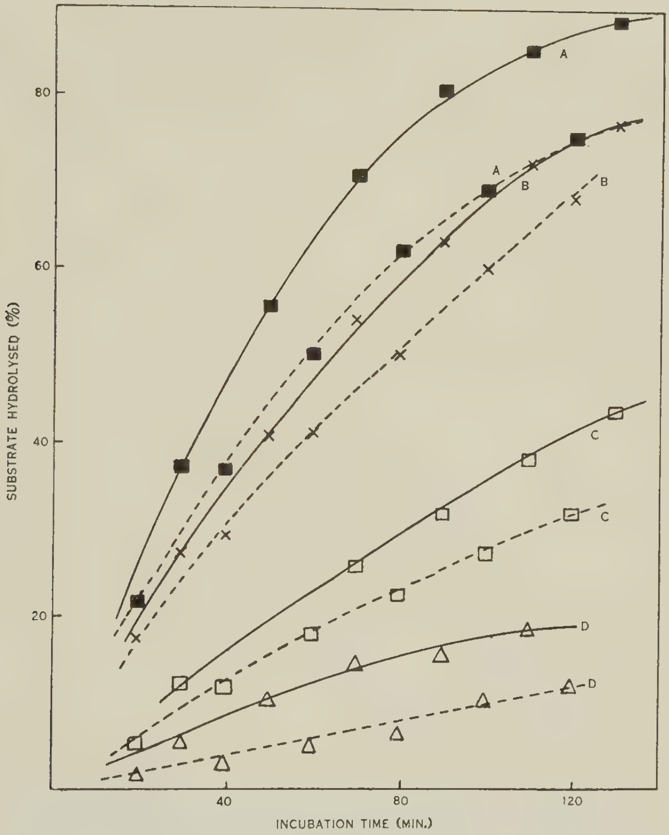

Fig. 1.—Hydrolysis of *p*-nitrophenyl-β-glucoside by various concentrations of crude *A. oryzae* enzyme. *A*, 450 μg./ml.; *B*, 300 μg./ml.; *C*, 120 μg./ml.; *D*, 50 μg./ml. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

The decomposition of salicin was followed at the same initial substrate concentration (5×10^{-4}M), determining liberated glucose in the same way and saligenin by the method of Folin and Ciocalteau (1927).

When it was desired to make a chromatographic examination of the products of enzymic action, the enzyme was first inhibited by boiling and the cooled solutions passed through a column of "Bio-Deminrolit" to remove buffer salts, *p*-nitrophenol, and enzyme material. The deionized material was then concentrated by distillation under reduced pressure, transferred to a small crucible, and evaporated at 30°C. in a current of air to a small volume (0.1-0.2 ml. from 100 ml.). The total time occupied in this process was 1-2 hr. so that there could have been no appreciable microbiological action.

The paper chromatography of sugars was carried out according to the methods of Jermyn and Isherwood (1949) using ethyl acetate + pyridine + water as the solvent.

III. RESULTS

(*a*) *Enzymic Hydrolysis of* p-*Nitrophenyl-β-glucoside*

Figure 1 shows the course of the hydrolysis of 5×10^{-4}M *p*-nitrophenyl-β-glucoside by various concentrations of crude *A. oryzae* enzyme. Figure 2 shows how this hydrolysis is modified by the presence of methanol and *n*-butanol.

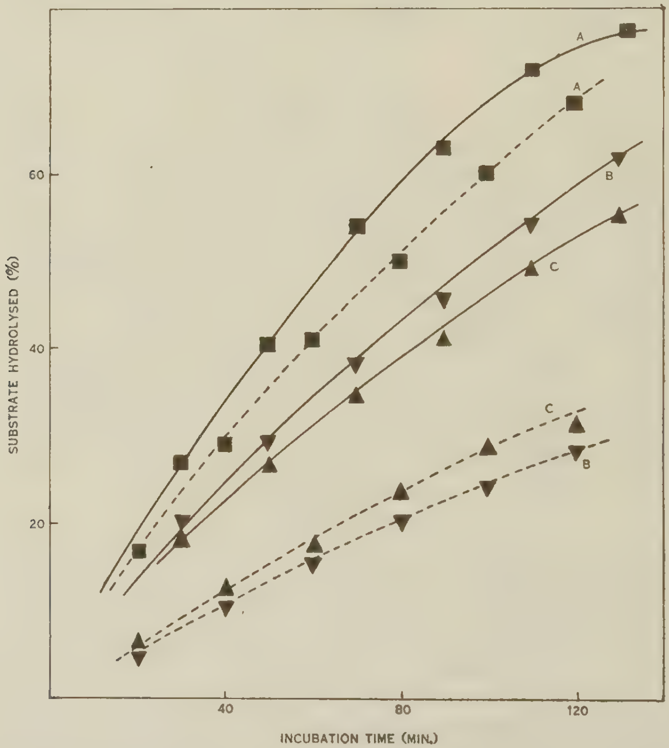

Fig. 2.—Hydrolysis of *p*-nitrophenyl-β-glucoside at a concentration of 300 μg./ml. of crude *A. oryzae* enzyme. *A*, no addition; *B*, 3M MeOH; *C*, 0.5M *n*-BuOH. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

The data of Figure 2 and other data not there inserted for the sake of clarity can be recalculated to give the curves of Figure 3 for the effect of various additions on the ratio of glucose liberated to *p*-nitrophenol liberated.

(*b*) *Enzymic Hydrolysis of Salicin*

Figure 4 shows the effect of methanol on the hydrolysis of salicin by the crude *A. oryzae* enzyme. Figure 5 is a recalculation of Figure 4 in terms of the glucose/*p*-nitrophenol ratio.

(*c*) *Other Observations*

When 5×10^{-4}M glucose was incubated at pH 5.0 and 37°C. with 300 μg./ml. of crude enzyme, there was no change in reducing values whether in the presence or absence of 3M methanol. Cellobiose (5×10^{-4}M) was hydrolysed rapidly by the enzyme under the same conditions and this hydrolysis was inhibited by methanol to about the same degree as that of salicin. The rate of hydrolysis of 5×10^{-4}M β-methyl glucoside (*c.* 1 per cent./hr.) was too low to have any effect on the course of experiments in the presence of methanol.

Hydrolysis of 5×10^{-4} *p*-nitrophenyl-β-glucoside by boiling 0.05N HCl showed exactly equivalent amounts of glucose and *p*-nitrophenol at all stages.

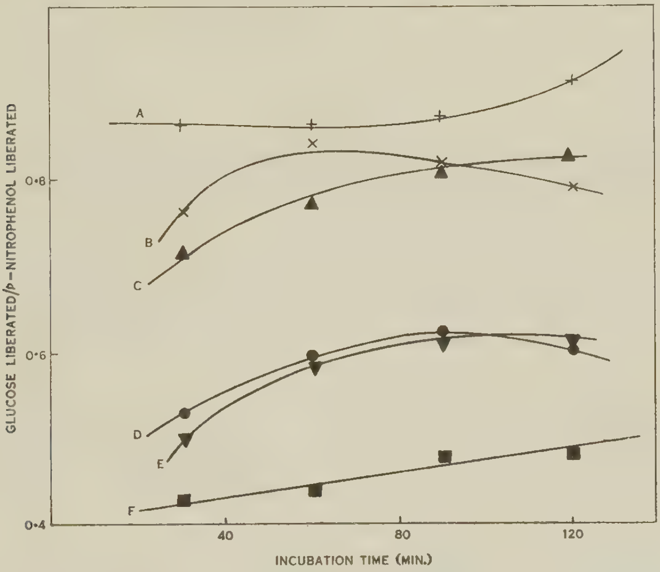

Fig. 3.—Variation in the ratio of glucose liberated to *p*-nitrophenol liberated on addition of various alcohols to the incubation mixture. *A*, no addition; *B*, 1M EtOH; *C*, 1M MeOH; *D*, 2M MeOH; *E*, 0.5M *n*-BuOH; *F*, 3M MeOH.

(*d*) *Chromatographic Experiments*

Salicin, cellobiose, and *p*-nitrophenyl-β-glucoside were incubated with the crude enzyme in the presence and absence of 3M methanol. The enzyme was inactivated when hydrolysis of the glucoside was half completed, as measured by the amount of aglycone liberated and the solution was examined by paper chromatography. The chromatographic observations are set out in Table 1. In addition to the substances listed, traces of sugars identified as galactose and mannose were invariably present, even when the enzyme was incubated alone. They apparently arise from breakdown of the polysaccharide material containing galactose and mannose which Jermyn (1952*a*) showed to be present in the crude mould enzyme. Other carbohydrate material was not detected on chromatograms from enzyme solutions incubated without substrate.

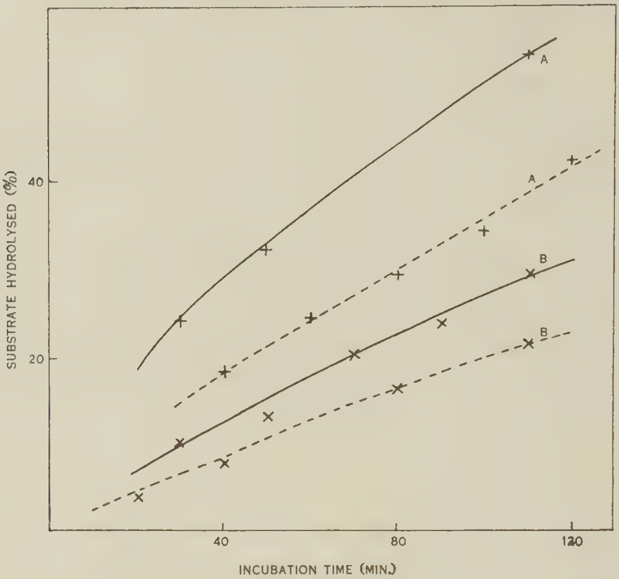

Fig. 4.—Hydrolysis of salicin at a concentration of 300 μg./ml. of crude enzyme. *A*, no addition; *B*, 3M MeOH. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

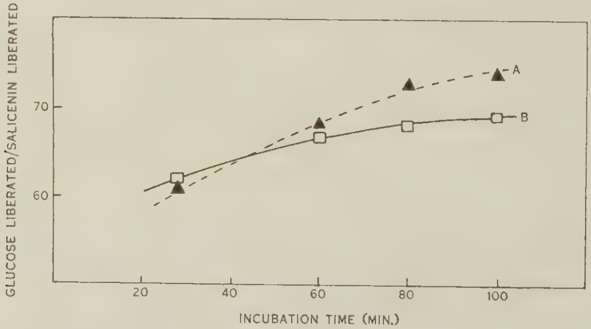

Fig. 5.—Ratio of glucose liberated to saligenin liberated in the experiments of Figure 4. *A*, no addition; *B*, 3M MeOH.

The substrate labelled "methyl glucoside" in Table 1 was identical with β-methyl glucoside in its chromatographic properties under all conditions tried. However, the difference between α- and β-methyl glucosides is so slight that a positive identification could not be made. The substances labelled "polysaccharide" were tested for by the use of numerous spraying reagents since it cannot be predicted *a priori* which reagents will react with the free reducing groups in such saccharides; ammoniacal silver nitrate and aniline phosphate were the most useful of these reagents. The non-formation of substances of the type glucosyl-glucosyl-aglucone could be demonstrated by spraying the developed paper with a solution of the enzyme and incubating. The liberation of *p*-nitrophenol and saligenin could be detected at a single locus — that of the corresponding simple glucosides.

When *p*-nitrophenyl-β-glucoside was incubated with the crude enzyme in the presence of 1.0M ethanol or 0.5M *n*-butanol, the formation of substances chromatographically identical with β-ethyl glucoside and β-(*n*-butyl)-glucoside respectively could be demonstrated by the technique used with the methyl glucoside. In view of the extreme difficulty of detecting these glucosides on paper, only the methanol system was further investigated.

TABLE 1

SUMMARY OF THE PAPER CHROMATOGRAPHIC ANALYSES OF CONCENTRATED ENZYME DIGESTS

+, Presence indicated; —, not detected

Substrate	Addition	Methyl Glucoside	Glucose	Cellobiose	Oligosaccharides* (mobile)	Polysaccharide* (immobile)
p-Nitrophenyl-β-glucoside	None	—	+	—	+	+
	3M MeOH	+	+	±	—	+
Salicin ..	None	—	+	—	—	+
	3M MeOH	—	+	—	—	+
Cellobiose ..	None	—	+	+	+	+
	3M MeOH	—	+	+	—	+

* The use of these terms is purely a matter of convenience and implies no final conclusions on the structure of the substances so labelled.

IV. DISCUSSION

The transfer of sugar residues by enzymes has been demonstrated in many systems and has been attributed (Wallenfels 1951) to a reaction of the type:

$$\text{Enzyme}-\text{OH}+\text{ROGl} \longrightarrow \text{Enzyme}-\text{OGl}+\text{ROH}\ldots\ldots\ldots(1)$$

$$\text{Enzyme}-\text{OGl}+\text{R}'\text{OH} \longrightarrow \text{Enzyme}-\text{OH}+\text{R}'\text{OGl}.\ \ldots.(2)$$

Where the enzyme glycoside complex reacts with water ($R' = H$) the result is hydrolysis. With an alcohol a new glycosidic linkage is formed. The enzymes of *A. oryzae* that produce methyl glucoside in solutions containing *p*-nitrophenyl-β-glucoside and methanol do not bring about a similar transfer of a glucose residue from salicin or cellobiose, a fact best explained by the theory of the presence of a number of β-glucosidases having different specificities (Jermyn 1952*b*). The order of effectiveness of different alcohols as competitors with water in reaction (2) (*n*-butanol > methanol > ethanol) differs from that found by Takano and Miwa (1950) for *A. niger* (methanol > butanol) but is the same as that of the enzymes of *P. chrysogenum*.

Takano and Miwa also demonstrated the transfer of a glucoside residue from *o*-cresyl-β-glucoside, a substrate closely related to salicin. Enzymes from different sources must be supposed to have widely different specificities for both substrate and acceptor. In *A. oryzae*, transfer of the glucosyl residue of *p*-nitrophenyl-β-glucoside to water and other acceptors is carried out by only one of eight enzymes present. This enzyme also acts upon salicin and cellobiose, but even if it catalyses some transfer to alcohols from these substrates its effect will be negligible compared with the total hydrolysis due to the non-transferring enzymes.

The presence of the alcohols appears to exert an inhibiting effect on the hydrolysis of both *p*-nitrophenyl-β-glucoside and salicin, with a more marked effect on the latter reaction. The inhibition is not very specific since methanol, ethanol, and *n*-butanol are about equally effective, and is not increased by the addition of more than a certain minimum amount of the inhibitor, methanol giving much the same inhibition at 1M, 2M, and 3M. The inhibition does not appear to have any direct significance for the transferase activity.

The β-glucosidases of *A. oryzae* can now be added to the list of enzymes that catalyse the synthesis of polysaccharide material by transglycosidase activity. Under the experimental conditions used the intermediate oligosaccharides formed in this synthesis appear to be present in very small concentrations. Since the products are those of a number of enzymes, it is unwise to attempt any theoretical interpretation of this observation. The upward trend of the curves of Figures 3 and 5 presumably means that the polysaccharides built up by transglycosidation are degraded by subsequent hydrolysis. This cannot be taken as a proof of the β configuration of the synthesized linkages; the crude *A. oryzae* material contains enzymes capable of hydrolysing all types of linkage tested. Even from cellobiose the new linkages synthesized would not necessarily be the β 1:4 of the cellulose chain and the formation of new linkages of the original type is in fact far from general in transglycosidation (Hehre 1951). Pazur and French (1951) have shown that the α-glucosidase of *A. oryzae* transforms 1:4 to 1:6 linkages.

V. ACKNOWLEDGMENT

The authors are deeply indebted to Mrs. M. C. Wilkinson for technical assistance.

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FACTORS INFLUENCING THE PAPER CHROMATOGRAPHY OF SOME ENZYMES OF *ASPERGILLUS ORYZAE* AND RELATED ENZYMES

By M. A. Jermyn*

[*Manuscript received September* 5, 1952]

Summary

The influence of a number of variables on the paper chromatographic behaviour of eight enzymes (proteinase, amylase, catalase, esterase, acid phosphatase, *p*-nitrophenyl-β-glucosidase, salicinase, and sucrase) present in the culture filtrate of *A. oryzae* has been investigated. The solvent systems used were mixtures of aqueous buffers and organic liquids. The nature and concentration of both the organic liquid and the buffer, pH, and the solute concentration strongly influence the results.

Methods are described for demonstrating the location of the enzymes on paper strips.

All the enzymes tested appear to have more than one component. The nature of these components is still obscure but it is unlikely that many of them are artifacts.

Multiple components can be demonstrated in certain commercial preparations containing enzymes with activities similar to those investigated in the *A. oryzae* preparation.

I. INTRODUCTION

The notes published by Reid (1950, 1951), Giri and Prasad (1951*a*, 1951*b*), and Cabib (1951), together with the more detailed papers of Simonart and Kwang Yü Chow (1951), Giri *et al.* (1952), and Cabib (1952) have explored various aspects of the paper chromatography of enzymes. Almost without exception these authors have devoted some part of their study to the separation of fungal enzymes. The high solubility under diverse conditions and resistance to denaturation of these enzymes make them very suitable subjects for chromatographic experiments. It has already been shown in this laboratory by the technique of filter-paper electrophoresis that many of the enzymes of *A. oryzae* consist of multiple components (Gillespie, Jermyn, and Woods 1952; Jermyn 1952*b*) and it seemed of interest to confirm these conclusions by another technique. Many types of solvent system are possible for the paper chromatography of enzymes; results using widely divergent systems would be almost impossible to correlate. Attention has therefore been concentrated on the systems containing water and an organic liquid which were first mentioned by Reid (1950).

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II. EXPERIMENTAL

(*a*) *"Mould Enzyme"*

The same batch of *A. oryzae* enzyme was used as in preceding papers (Jermyn 1952*a*, 1952*b*). It was not identical with that used by Gillespie and Woods (1952), who have observed considerable variation between batches of the material.

(*b*) *Paper Chromatography*

An all-glass apparatus for descending chromatography was used. The solvent mixture was poured into both the bottom of the tank and the solvent trough and the whole allowed to equilibrate for some hours. Identical composition of both lots of solvent was found to be extremely important in obtaining reproducible results. Whatman No. 54 paper was used throughout this study in a standard strip of 46 by 15 cm. Enzyme solutions were spotted along a line 7.5 cm. from the top of the strip, which was air-dried for 15 min. and transferred to the tank for development. On completion of development the strip was removed from the tank, dried at room temperature in a rapid current of air for 15-30 min., and kept in a deep-freeze unit at –20°C. till required. It was found that all enzymes studied could be held at this temperature overnight without losing much activity.

(*c*) *Detection of Enzymes*

Reid (1950) and Giri and Prasad (1951*a*) have used the agar-plate method for detecting the position of enzyme proteins. It appeared more satisfactory to follow Simonart and Kwang Yü Chow (1951) and Cabib (1951, 1952) in allowing the enzyme to react with its substrate on the paper strip as a method detecting it. By suitable choice of substrate the tedious incubation period necessary in the agar-plate method can be avoided. Diffusion and other effects can blur apparent separations and cause distortion of spots if long incubation periods are employed for the direct reaction on paper; the methods used have therefore been chosen to give a maximum chromogenic effect in 30-60 min.

Reagents were uniformly applied to the paper with a fine spray. Where the reagent had difficulty in penetrating evenly, as with starch and azocasein solutions, the paper was sprayed rather more lightly back and front. The mean optimum pH for the action of most of the *A. oryzae* enzymes being known, the pH of the reagent spray was so adjusted that the pH of an extract of the paper after a test spraying was in the correct range. This often necessitated the use of rather concentrated buffers, but checking revealed that the activity of none of the enzymes studied was markedly affected by the ionic strength of the medium.

The average laboratory temperature during the course of the experimental work was 22-25°C. and it was found that the most satisfactory method of incubation was simply to lay the sprayed strips horizontally on clean, non-adsorbent paper for 30-60 min. Hanging the strips in a humidity chamber did not lead to a marked improvement of results. Raising the temperature of incubation led to marked over-development unless very short incubation periods were used; it then became correspondingly difficult to stop the enzymic reaction at intermediate stages. Inactivation of the enzyme was the limiting factor in any attempt to overcome these difficulties by reducing the concentration at which the enzyme was applied to the paper. The conditions of enzyme and reagent concentration used for any enzyme are those that have been found empirically to give ease of handling and minimum sensitivity to small changes in conditions.

(*d*) *Enzyme Reagents*

Eight enzymes of *A. oryzae* have been used in this study. An outline of the conditions for the detection of each enzyme is given below.

(i) p-*Nitrophenyl-β-glucosidase.*—Preparation of the *p*-nitrophenyl-β-glucoside reagent has been previously described (Jermyn 1952*b*).

(ii) *Salicinase.*—The spraying reagent was 1 per cent. salicin solution buffered so that the final pH on the paper was about 5.0. After incubation the paper was dried at 105°C. and sprayed with 0.1 per cent. *p*-anisidine hydrochloride in ethanol. On heating at 105°C. the areas where glucose had been liberated by enzymic reaction appeared as yellow to brown with a bright green fluorescence under the ultraviolet lamp (Hough, Jones, and Wadman 1950). This reagent was scarcely affected by non-reducing glucosides or proteins.

(iii) *Sucrase.*—As for salicinase except that the spraying reagent was 1 per cent. sucrose solution.

(iv) *Amylase.*—The paper was sprayed with 0.5 per cent. starch solution buffered to give a final pH of 6.0 and after incubation dried at 105°C. The starch was found to be strongly adsorbed on the paper and the enzyme could be detected as colourless to violet areas against a blue-black background by passing through a bath of 0.005N iodine and blotting.

(v) *Catalase.*—The paper was sprayed with 0.5 per cent. hydrogen peroxide solution, and after 20 min. sprayed again with the ferric chloride-ferricyanide reagent of Feigl and Frankel (1932). Catalase appeared as yellowish white areas against a bright blue background. This test failed in the presence of phosphate.

(vi) *Acid Phosphatase.*—The paper was sprayed with a saturated solution of calcium phenolphthalein phosphate in acetate buffer adjusted to give a final pH on the paper of 5.0. After incubation the paper was sprayed with 5 per cent. trisodium phosphate solution. This maintained an alkaline reaction on the paper for long periods, showing the presence of acid phosphatase as a pink area on a white background.

(vii) *Proteinase.*—The modified Gram-staining method of Simonart and Kwang Yü Chow (1951) gave very low contrast between areas where the *A. oryzae* proteinase was present and absent. The azoproteins and in particular the azocaseins which have been recommended for the estimation of proteinase (Charney and Tomarelli 1947) appeared to be more promising substrates. Those in common use give their maximum colour at an alkaline reaction and on paper the coloured alkalized proteins are subject to fading and discoloration. A satisfactory substrate was found in *p*-dimethylaminobenzene azocasein. This substance shows a bright purple-red colour in acid solution and on precipitation with acid reagents, which is much more intense than the brownish colour in alkaline solutions. It has two solubility ranges—between pH 1.0 and 4.0 and above 6.0—and is thus a suitable substrate for pepsin. The properties of certain azocaseins investigated are summarized in Table 1. It appears that an ionizable substituent in the aromatic ring confers acid solubility on an azocasein; if this substituent is a basic group the colour is more intense in acid than in alkaline solution.

The spraying reagent used was a 2.5 per cent. solution of *p*-dimethylaminobenzene azocasein adjusted to give a final pH of 7.0 or 1.5 (pepsin). After incubation the paper was passed through a bath of 10 per cent. trichloracetic acid and then washed for 30 min. in running tap water (pH *c.* 7). The proteinase was revealed as white areas on a pink background.

(viii) *Esterase.*—Included here are those enzymes only which will hydrolyse *p*-nitrophenyl acetate; other more specific enzymes if present were not studied. The *p*-nitrophenyl acetate (0.01 per cent. in 20 per cent. ethanol buffered to give a final pH of 7.0) was at first used itself as the spraying reagent but spontaneous breakdown on the paper gave rise to a high level of background colour. Acetyl derivatives of phthaleins were found to be much more satisfactory substrates; comparative tests with the two reagents showed an identical pattern of esterase distribution. The preferred reagent was a 0.01 per cent. solution of diacetyl fluorescein in 50 per cent. acetone buffered to give a pH of 5-7 on the paper. The acetone evaporated sufficiently rapidly not to interfere with the enzyme action, which could be followed under the ultraviolet lamp by the development of fluorescent areas. The acetyl derivatives of phenolphthalein (pink areas after brief exposure to ammonia vapour), metacresol purple, and chlorphenol red were also satisfactory, although less sensitive, substrates. The substitution of both positions ortho to the acetoxyl groups gave substances unaffected by the enzyme, the acetyl derivatives of eosin and bromphenol blue, for instance, being ineffective as substrates.

III. Chromatography of the A. oryzae Enzymes

(*a*) *Flow of Various Solvents Down the Paper*

It is generally agreed that the attainment of equilibrium between paper and solvent before the start of development is necessary for reproducible results (see Smith 1952 for a summary). In the present study, however, long equilibrium periods led to inactivation of the enzymes spotted on the paper. The differential volatility of the constituents of the solvent system used meant that the paper could not be soaked in the solvent before use. In these circumstances, acceptance of incomplete equilibration offers the only prospect of reproducible results.

Mixtures of ethanol or acetone with buffers were allowed to travel a certain distance down the paper, the apparent solvent front marked, and the paper dried and sprayed with an indicator solution. It was found that there was a sharp boundary between two regions of different pH. The factors determining the position and nature of this boundary were:

(i) *Concentration of Buffer.*—A solvent containing 40 per cent. acetone and 60 per cent. of 0.1M acetate buffer of pH 5.0 gives two regions; if the solvent is allowed to flow 20 cm. past the starting line, the boundary is 15 cm. in advance of the line. When this solvent is modified by decreasing the concentration of buffer, the boundary approaches the solvent front, coinciding with it at about 0.02M.

(ii) *Concentration of Organic Component.*—In general, the higher the proportion of this component, the lower the buffer concentration needed to produce the boundary phenomenon.

(iii) *Nature of the Buffer.*—In general, the pH of the region nearest the solvent front is more acid if the "organic" component of the buffer is acid and more basic if the "organic" component is basic. These conditions are attained for example with acetate and aniline buffers respectively. Inorganic buffers such as phosphate do not produce the double front phenomenon so readily as organic buffers.

Another phenomenon that can be demonstrated when the results of experiments on enzyme travel are considered is the existence of a third region in front of the apparent solvent front. When the distance of solvent travel is 20 cm., enzyme spots can be located in positions up to 10 cm. in front of the apparent solvent front. The tendency for spots to appear in this position is greater at lower pH values and depends on the enzyme studied; it is evidence for the existence of an invisible flow of liquid through the paper ahead of the apparent solvent front. R_F values greater than 1.0 have been noted when ionic solutes are being separated on paper with water-miscible solvents (Lederer 1950) and show that no simple picture of the processes involved in paper chromatography is adequate.

Reid (1951) speaks of enzymes travelling at "water" and "solvent" fronts; the phenomena indicated are apparently counterparts of those discussed above.

(*b*) *Behaviour of Enzymes as Solutes*

(i) *General.*—It was at once established that the enzyme proteins of *A. oryzae* move on the paper under the general conditions set out in the experimental section. The enzyme spots may be immobile, move in the solvent front or the "false front" (boundary between the two regions of different pH), or occupy various intermediate positions.

Where an enzyme neither remained at the starting line nor moved in one of the fronts it was found that as the solvent front advanced the enzyme spot lagged further and further behind the front until it was a certain definite distance behind, in which position it remained during further development. When

TABLE 1

PROPERTIES OF SOME AZOCASEINS

Aromatic Amine Diazotized	Solubility Ranges (pH)			Colour			Action of Enzymes (colour in solution after precipitation with CCl_3COOH) under Identical Conditions		
	Alkaline	Intermediate Range	Acid	Solid (acid form)	Solution (acid)	Solution (alkaline)	Mould Enzyme Acid Colour	Mould Enzyme Alkaline Colour	Pepsin
p-Dimethyl amino aniline	>6·0	Slightly soluble minimum 5·0	4·0-1·0	Maroon	Red	Dark brown	++++	+++	++
J-Acid (6 amino-α-naphthol-3-sulphonic acid	>6·5	Slightly soluble; insoluble at 5·0	2·5-1·0	Purple	Reddish purple	Brownish purple	+	+	++
Arsanilic acid	>6·5	Slightly soluble minimum 4·0	3·0-1·0	Light yellow	Light yellow	Orange	+	++	+
p-Phenylene diamine (monodiazotized)	>6·5	Slightly soluble minimum 6·0	4·5-1·0	Brick red	Brick red	Light brown	++	+++	+
p-Amino benzoic acid	>4·5	Slightly soluble; insoluble 2·0-3·0	>1·0< narrow range	Dark yellow	Light yellow	Yellow-brown	++	+++	
β-Naphthylamine 6 : 8 disulphonic acid	>5·8	Slightly soluble slowly falling off to 1·0	—	Orange	Orange	Reddish brown	+++	++++	

the solvent front had travelled 20 cm., the enzyme spots had usually attained maximum distance behind the front and this distance was chosen as a standard distance of travel. In practice a second line was ruled 20 cm. in advance of the starting line and the paper removed when the solvent had reached this second line.

When solvents containing low concentrations of acetone are employed, there is a strong tendency for the bulk of the protein present to be carried in the advancing liquid front; at higher concentrations it tends to be carried in the "false front," in cases where this is formed. Under any given conditions of pH and concentration of the buffer, a number of different enzymes are found to travel the same distance down the paper; the position they occupy is found to be identical with that of the bulk of the protein (located by bromphenol blue staining). The frequency of this identification varies from enzyme to enzyme. The position of the main protease component for instance almost always coincides with the "protein spot"; the esterases are almost always distinct.

(ii) *Inactivation and the Effect of Temperature.*—As a dry powder, the *A. oryzae* enzyme mixture is stable indefinitely at room temperature. When dried out of solution on filter paper it is, however, extremely unstable; the activity of most of the enzymes begins to decrease markedly after 30 min. at room temperature (20°C.) and although they can be preserved for 24 hr. at –20°C. some loss of activity is detectable. The periods of handling and incubation of the enzymes on the paper were kept to a minimum and the main effects of denaturation therefore occurred during the actual development period.

The *A. oryzae* enzymes in solution are relatively resistant to changes in temperature, and the same results were obtained with otherwise identical chromatograms developed at temperatures between 1°C. and 30°C. In view of these facts no attempt was made to control temperature during these experiments, which were carried out at room temperature with the tank atmosphere saturated at the temperature employed.

(*c*) *Variation of the Organic Component*

The requirements for the organic component limit the choice to a relatively few substances. It must be readily volatile to avoid prolonged drying periods; neutral (pyridine and acetic acid at useful concentrations produced complete denaturation of all enzymes tested); relatively non-viscous to avoid prolonged development; miscible with water over a wide range of concentrations; and relatively inexpensive.

It is impracticable to check possible compounds under all conditions. Table 2 shows the effect of a number of organic solvents in admixture with 0.1M acetate buffer of pH 5.0 on the travel of three enzymes. It is uncertain on what basis various organic liquids ought to be compared; in Table 2 they have been compared at the same mole fraction (that of 40 per cent. acetone).

It appears that acetone and methanol are most satisfactory for the purpose and this appears to be true for conditions other than those listed in the table. For many separations of non-enzymic proteins where inactivation is not so important and longer development times are possible, *tert*-butyl alcohol is a satisfactory solvent.

The rest of the work reported in this paper has been carried out using acetone as solvent.

TABLE 2

EFFECT OF VARIOUS ORGANIC SOLVENTS ON THE PAPER CHROMATOGRAPHY OF THREE ENZYMES OF *A. ORYZAE*

Mole fraction of organic component 0.142, buffer: 0.1M acetate, pH 5.0. Crude *A. oryzae* enzyme spotted on as 10 per cent. solution, final spot size, 0.5 cm. diam.

Organic Component	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)			Protein Staining After 20 cm. Solvent Travel (bromphenol blue)
	Catalase	Amylase	β-Glucosidase	
Acetone	12·5-14·5	4·5-11	±0·5	7-16
		11·5-15	10·5-16	
Methanol	16-18	±0·5, 10·5-15,	±0·5	15·5-20
		15-19·5	12-16·5	
		Trail 5-10		
Ethanol	14·5-16	18-20	0-2	
	Two short, parallel streaks	Trail 15·5-18	17·5-20	14·5-20
n-Propanol	14-16	18·5-20	−1·5-2·5	18·5-20
	As for ethanol	Trail 13-18·5	17·5-20	
*iso*Propanol	13-15·5	16-20	17-20	
	Very faint			
tert-Butanol	Inactivated	13·5-20	17-20	19-20
Dioxan	13·5-15·5	18-20	±0·5	±0·5
		Trail 15-20	15·5-20	14-20
Ethyl cellosolve	13-15	±0·5	19·5-21·5	±0·5
		19·5-21		19-20
Tetrahydrofuran ..	15-17	19-20	±1	18·5-20
	Almost inactivated		11·5-17	

(*d*) *Variation of Buffer*

In Table 3 are listed the effects of variation in buffer on the separation of eight enzymes under a single set of conditions. Only two types of buffers—$Na^+/(AH + A^-)$ and $(B + BH^+)/Cl^-$—were studied, so that effects due to changes in ions other than the buffering species were eliminated.

(*e*) *Variation of Buffer Strength*

Table 4 shows the effect of varying the concentration of acetate buffer of pH 5.0 admixed with 40 per cent. of acetone between 1.0 and 0.002M.

The increasing definition of the spots with decreasing molarity of buffer cannot be extrapolated to extremely low concentrations. When a mixture of acetone and distilled water was used as the developing solvent, the sole ions in the medium coming from those present in the crude enzyme solution (pH 6), the spots became very diffuse and streaky. If the solution of crude enzyme was replaced by one made from a sample of enzyme which had been deionized by passing it through an ion-exchange column, the various enzymes appeared as streaks extending from the starting line to the solvent front.

(*f*) *Variation of Enzyme Concentration*

The effect of variation in the concentration of solute is shown schematically in Plate 1 for an enzyme preparation having a single component and for one having two components. It is noteworthy that the position of the enzyme front remains practically constant and that the enzyme concentration controls the amount of tailing. However, it is not always possible to resolve an enzyme which appears after development as a long trail into a discrete spot or spots by reducing the concentration at which it is applied to the paper. Under unfavourable conditions the enzyme will remain as a streak down to the limits of detection.

It is important to note that the streaking effect is never entirely eliminated. Experiments with the β-glucosidase and esterase, where the colour is released directly by the enzyme, show discrete centres of colour appearing on incubation which are later linked by a diffuse trail. When conditions are such that the small amounts of enzyme responsible for these trails are inactivated, the residual enzyme is detected as sharply outlined spots. This type of effect is particularly noticeable with horse-radish peroxidase (Jermyn 1952*c*). Suitable concentrations for the application of given enzymes were determined by trial and error and often had to be further modified after changing the conditions of development.

(*g*) *Variation of pH and of Concentration of the Organic Component*

The effect of varying these two factors was checked for six enzymes in a single series of experiments. The results are tabulated in Table 5.

Two of the *A. oryzae* enzymes (catalase and acid phosphatase) could not be detected after development with solvents containing the citrate-phosphate buffer. In Table 6 are tabulated the effects on these two enzymes of gradually increasing the concentration of acetone in admixture with acetate buffer.

(*h*) *Other Factors*

The *A. oryzae* enzyme solutions were always applied in such a way as to give a round spot of 1 cm. final diameter. None the less the shape of the final spot of enzyme after development was often characteristic. Catalase gave a heart-shaped spot with the point directed backwards. In certain solvents favouring denaturation of the catalase the spot appeared as two streaks parallel to the direction of solvent flow with a clear space between them. The acid phosphatase spot was always very small and showed no evidence of lateral diffusion; unfavourable solvent systems changed it into a streak no more than 2 mm. wide. These appearances can be correlated with the distribution of the enzyme

TABLE 3

EFFECT OF VARYING THE NATURE OF THE BUFFER ON THE PAPER CHROMATOGRAPHY OF EIGHT ENZYMES OF *A. ORYZAE*

Acetone 40 per cent. by volume; buffer: 0.1M, pH 5.7

Buffering Agent	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)							
	Catalase	Acid Phosphatase	β-Glucosidase	Esterase	Amylase	Protease	Salicinase	Sucrase
Pyridine	10·5-15·5	4-10·5	1·5-6·5 11-12 Very faint	±1 6-11 12·5-14·5	±1 3·5-10·5 10·5-12	±0·5 1·5-11	3-5·5 15-17·5	±1 5-12·5 15·5-18
Aniline	13·5-17	15·5-17	12·7-14 15·5-17	±1 13·5-16·5 18·5-21·5	±1 7-14·5 14·5-16·5	−1-3 Trail 10-17	11·5-15 16-18	12-14 15-19
Ethylene diamine ..	12-17	16-18	0-7 Almost inactivated	±0·5 Trail 13·5-16 trail 17·5-20·5	5·5-17 (streak) Trail 0·5-5	±0·5 9-18 Trail 1-9	5·5-10 15-17 Trail 0-5·5	13·5-19·5 Trail 4-13·5
Histidine	10-15·5	3-9·5 Almost inactivated	Trail 4-9 Trail 14-17	Fails	−1·5-10·5	−1-12·5	0-6 16·5-18 Almost inactivated	Trail 10·5-12·5 12·5-14 15-17
Ethylene diamine tetracetic acid ..	Fails	15·8-17·8	0-21 (streak)	±0·5 11-21 Almost inactivated	7-17 (streak)	8·5-16	15-17 Long trail back to 6	7·5-13 14·5-18
Maleate	12-14	15·5-17·5	9-16·5	−0·5-1·5 11-16·5 18·5-21	11-16	10-17	8·5-13 15-18	±0·5 Trail 6-13·5 14·5-16 16·5-19

TABLE 3 (*Contined*)

Buffering Agent	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)							
	Catalase	Acid Phosphatase	β-Glucosidase	Esterase	Amylase	Protease	Salicinase	Sucrase
Phthalate	12-15·5	17-19	15·5-18 (faint)	±0·5 10-17 17-20	7-17 (streak centred at 15)	0-2 6·5-18	Trail 7-14 14-18	0-2 3·5-9·5 Trail 13·5-16 16-19 19-22
Phosphate	Fails	Fails	6-11·5	±0·5 11·5-15·5 17-20	Trail 13-15	6-16	4-11 12·5-15·5	±0·5 Trail 5-11·5 Trail 13-16
Citrate	Fails	Fails	0-8·5 10·5-16 Very faint	±0·5 9·5-14 18-20	0-3 3-11·5 11·5-15	5-11·5 11·5-15	2-7·5 Trail 11-14	±0·5 Trail 4-11·5 12-16 16-18·5

in the original spot—acid phosphatase being adsorbed on the paper at the actual point of application, and catalase being carried out to the boundary of the expanding spot. Careful spraying of spots of enzyme solution freshly applied to the paper confirmed this hypothesis.

Table 4

EFFECT OF VARYING CONCENTRATION OF THE BUFFER ON THE PAPER CHROMATOGRAPHY OF THREE ENZYMES OF *A. ORYZAE*

Acetone 40 per cent. by volume; buffer: acetone, pH 5.0

Buffer Concentration (Molarity)	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)			Protein Staining After 20 cm. Solvent Travel (bromphenol blue)
	Catalase	Amylase	Esterase	
1·0	Fails	13-16	±0·5 14-17	14-17
0·2	13-15	5-11·5 11·5-15	±0·5 10-13 13-15·5	5-16
0·04	13·5-15·5	9-17·5 17·5-20	±0·5 Trail 15-18 19-20	11-20
0·01	13-16	±0·5 9-11 11-13·5 14-17·5 Trail	±0·5 Trail 11-14 17·5-20	14-18·5
0·002	12-15·5	11·5-14 14-17·5	0·5-1·5 12-13·5 13·5-17 18-19·5	12·5-20

Cabib (1952), who tested the behaviour of purified serum albumin and globulin and mixtures of the two using ethanol-buffer solution mixtures as chromatographic solvents, found that the two proteins behaved differently according to whether they were separate or mixed. No trace of this effect has been detected with the enzyme proteins of *A. oryzae*. Various fractions have been tested in which certain of the enzymes have been concentrated and others partially removed (Jermyn 1952*b*) but without effect on the chromatographic behaviour of the enzymes.

IV. Chromatography of Enzymes from Other Sources

A series of enzymes with the same general activities as some of those present in *A. oryzae* were tested under the conditions that had proved most successful for separation of the corresponding *A. oryzae* enzyme into more than one component. The solutions of enzyme were initially made up to the same nominal concentration that had been used for the *A. oryzae* enzyme and then suitably diluted if necessary to give discrete spots on chromatography. The enzymes were mostly samples from the laboratory shelves of unknown age and history. Their "solutions" were centrifuged to remove insoluble material. The results are summarized in Table 7.

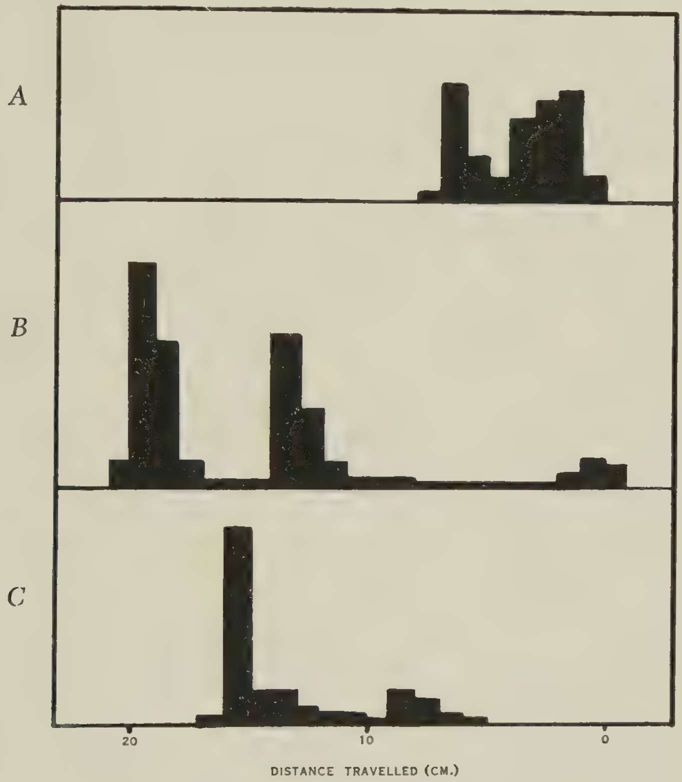


Fig. 1.—Distribution of certain enzymes along paper strips after paper chromatography. The enzyme activities are in arbitrary units.

A, *p*-nitrophenyl-β-glucosidase: 70 per cent. acetone; citrate-phosphate buffer, pH 6.0

B, esterase: 40 per cent. acetone; 0.01M acetate buffer; pH 5.0.

C, proteinase; 40 per cent. acetone; citrate-phosphate buffer; pH 5.0.

V. Quantitative Determination of Enzyme Distribution

To check the view that the multiple enzyme spots observed correspond to a real distribution of enzymes and are not artifacts of spraying, developed strips were cut into 1 cm. widths and the distribution of enzyme down the paper estimated directly. The results are shown in Figure 1.

TABLE 5

EFFECT OF VARYING pH OF THE BUFFER AND PROPORTION OF THE ORGANIC COMPONENT ON THE PAPER CHROMATOGRAPHY OF SIX ENZYMES OF *A. ORYZAE*

Buffer: McIlvaine (citrate-phosphate), 0.01M. Esterase, β-glucosidase, and protease applied as spots of 10 per cent. solution, amylase as 4 per cent., sucrase and salicinase as 1 per cent.

Acetone Concentration (% by volume)	pH	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)					
		Esterase	β-Glucosidase	Protease	Sucrase	Salicinase	Amylase
0	4	6-7 14-19·5	Fails	Trail 15-20·5	21-25·5	0-21	19-26·5
	6	0-6·5 14-20	15·5-19	16-20	±0·5 1·5-2·5 Trail 9-10·5 11-22	24-29	9-14 18·5-28·5
	8	Fails	Trail 19-20	−3-29	0·5-5·5 6·5-10 16-25 26·5-30	24-29	9-14 18·5-28·5
10	4	2-4 15-20	Fails	15-20·5	20·5-22·5	Trail 17-21	±0·5 7·5 Trail 20-26
	6	Trail 15·5-21	Trail 18·5-20	17-20·5	Trail 18-19·5 20·5-22	±0·5 10-12 18-21·5	±0·5 16·5-21
	8	Fails	Trail 20-21	11-26	16·5-20 20·5-21·5 23-27	20-27	0-4 19-28
20	4	±0·5 19·5-23	Fails	18-21	21-24	Trail 17-22·5	±0·5 19-30
	6	−3-2·5 12-21	15·5-18·5 19-20·5	15-20·5	Trail 5-7 Trail 10-17 18-20·5 22-23	Trail 16-20·5	16·5-20
	8	0-1·5 15-20·5	20-21	11-21	19·5-24	17-19 19-25	10·5-15 21-25
30	4	Trail 18·5-21·5	Fails	16·5-21	21-27 Very faint	Trail 16-21	10·5-15 16-23
	6	−4-3 15-19 19-21·5	Trail 21·5-23·5	16·5-20·5	±0·5 Trail 7-11 Trail 19·5-21	Trail 19-21·5	21-22
	8	0-2·5 15-22	18-22	11-20	20-22	15·5-18·5 22·5-26·5 29·5-31·5	9-11 18-23

TABLE 5—*Continued*

[illegible]	pH	[illegible]					
		Lactase	β-Glucosidase	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	4	[illegible]·5 15–18·5 [illegible]	Fails	14·5–18	Fails	4–8 13–21	2–4·5 14–19
	[illegible]	[illegible]	[illegible]	15–20	11–20 Faint	16·5–20·5	Trail 14–18·5
	[illegible]	[illegible]	[illegible]	3·5–23	14·5–23	4·5–23·5	12·5–19
[illegible]	[illegible]	[illegible]	[illegible]	12–17·5	Fails	7·5–11 Trail 15–18	2–9 11·5–14 15–17
	[illegible]	[illegible]	[illegible]	12–20	5–13 16–22·5	Trail 11–18 18–20	−0·5 4–18·5 18·5–20
	[illegible]	[illegible] 16–20 [illegible]	[illegible]	Trail 18–27	15·5–22·5	12–15 16·5–22·5	15·5–21·5
[illegible]	[illegible]	[illegible]	[illegible]	0–13·5	Fails	4–6·5 13·5–16	−2·5–8 10–12 13–15
	[illegible]	[illegible]	[illegible]	2–22	4–10·5 17·5–21	0–23 multiple centres	−0·5 5·5–17
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible] multiple centres	[illegible]	[illegible] 1–16
[illegible]	[illegible]	[illegible]	[illegible]	−1 Trail 12–16	11·5–18 15–19 [illegible] spot	−1 14–16	−0·5 4–6 8–11·5 13–16·5
	[illegible]	[illegible]	[illegible]	−3–4	−3–2	−1·5–3·5	Faint
	[illegible]	[illegible]	[illegible]	−2·5–4 5–16	4–8 9–13 14·5–16·5 17–20·5 21–22	Multiple spot centres at 3, 5, 9, 12·5, 15·5, 21·5, 27·5	−0·5 3·5–17

VI. CONCLUSIONS

The conclusions emerging from the experimental portion of this paper may profitably be summarized (Table 6) before proceeding to discuss them.

Other conclusions may be drawn as follows:

(*a*) All the factors listed in Table 8 interact so that the pattern of enzyme behaviour cannot be accurately predicted in a new environment.

(*b*) Each enzyme behaves as an independent species.

(*c*) Where an enzyme activity is the sum of the activity of several components there are gradations in behaviour between cases where the properties of these components are as widely different as those of unrelated enzymes (esterase) and cases where their properties are so similar that only occasionally are separations possible. These conclusions apply only to the peculiar conditions of paper chromatography.

(*d*) The concept of an absolute R_F value has no validity in the type of system studied in this paper.

(*e*) The results are reproducible.

TABLE 6

EFFECT OF VARYING THE PROPORTION OF ACETONE ON THE PAPER CHROMATOGRAPHY OF TWO ENZYMES OF *A. ORYZAE*

Buffer: acetate, 0.1M, pH 5.0

Acetone Concentration (% by volume)	Distance Travelled by Enzyme Spot After 20 cm. Solvent Travel (cm.)	
	Catalase	Acid Phosphatase
40	13-16·5	18-21
60	12-16	15-16·5
70	8·5-13	−3-2
80	Trail 7-9	−3-1
	9-13	
90	−1·5-0·5	
	1-6	

VII. DISCUSSION

(*a*) *General*

It now seems established that when suitable solvent systems are used enzyme proteins will serve as solutes for conventional paper chromatography. Too few results are as yet available to allow valid generalizations to be drawn and the results of this and other papers cannot be properly compared. Giri *et al.* (1952) have shown profound species differences in what is nominally the same enzyme, e.g. they showed that rat and human serum phosphatases differed chromatographically from chick serum phosphatase. It seems probable that enzymes of different specificity from one source, for instance exo-enzymes of a fungus, may be more nearly related in the relevant physical properties than enzymes of the same activity from different sources.

The theory and general application of precipitating solvents of the organic liquid-aqueous buffer type to the differential separation of proteins have been given by Cohn *et al.* (1950). The factors they find to be important—pH, ionic strength, nature of the buffering ion, concentration of the organic component—are equally important in paper chromatography with these solvents. Their conditions for minimum solubility at a given concentration of the organic component are a low but definite ionic strength, a definite pH, and the presence of given ions. In paper chromatography these are the conditions not for minimum distance of travel, but for maximum definition of enzyme spots and sharpness of separation of multiple components. It is only under conditions defined by a certain set of values that given enzyme proteins will have the sharply individual physical properties on which the adsorption-partition mechanism can operate to give differential behaviour. The adsorption-partition mechanism is itself governed by pH, ionic strength, solvent concentration, and other factors.

In view of the importance of the pH of a precipitating solvent, it is surprising that a study of its influence has so far been neglected by workers on the paper chromatography of enzymes. Cabib (1951), who used phosphate buffers of pH 7, stated that the pH of the buffer had no influence on the results. Other workers have claimed satisfactory results using water-acetone mixtures quite unbuffered. It is not possible at present to reconcile this behaviour with that of the *A. oryzae* enzymes. These enzymes vary in the degree to which their behaviour changes with change of pH; the yeast enzymes of Cabib (invertase and phosphoglucomutase) may have been relatively indifferent to pH. The enzyme preparations of other workers may may have contained enough salts to give an ionic strength where well-defined enzyme spots would appear after development.

Reid (1951) has contested the movement of enzyme spots in solvents containing more than 40 per cent. acetone. The results of the present paper confirm the report of Giri and Prasad (1951*a*) that solvents containing more than 40 per cent. acetone are effective. With catalase there is even some movement in 90 per cent. acetone.

Two further points of disagreement with other workers are that distance of travel does not fall off with increasing concentration of the enzyme (Giri *et al.* 1952) and that there is no sign of interaction between proteins (Cabib 1951, 1952). The observations of Jermyn (1952*b*) on the similar chromatographic behaviour of the *p*-nitrophenyl-β-glucosidases in both crude and purified enzyme material were confirmed in the present study by observing the behaviour of these enzymes and of the esterases in many different fractions of the crude *A. oryzae* material. If a given enzyme component was present it occupied exactly the same position on the chromatogram whatever the preparation studied. This contrasts with the results of Cabib and of Giri *et al.* Again no more than a tentative explanation can be given—that the proteins excreted by *A. oryzae* as exo-enzymes are so similar that they do not react together in the way that the dissimilar proteins present in tissue extracts or serum might be expected to do.

TABLE 7

COMPARISON OF CERTAIN ENZYMES OF *A. ORYZAE* WITH COMMERCIAL ENZYMES HAVING THE SAME APPEARANCE

(*a*) Amylases

Acetone 40 per cent; buffer: 0.1M pyridine/HCl, pH 5.7

Enzyme Source	Appearance After Solvent Front has Travelled 20 cm.
U.S.P. Diastase	Spot at ±0·5 cm.; short forward trail
“β-Amylase”	Spot at ±0·5 cm.; short forward trail; well-defined spot at 14·5-16 cm.
“α-Amylase” Same source as β-amylase	Streak −0·5-2 cm.; streak 3-8·5 cm.; well-defined spot at 14·5-16 cm.
Analytical malt diastase	Spot ±0·5 cm.; long forward trail to 9 cm.; second major spot 12-15 cm.
“Amylase”	Spot at ±0·5 cm.
Human saliva	Spot at ±0·5 cm.
Amylopsin	Spot ±0·5 cm.; short forward trail
“Diastase”	Spot at ±0·5 cm.; streak 2-10·5 cm., spot 12-14 cm.
Taka-diastase	Spot at ±1 cm., streak 2-12 cm., spot 12·5-13·5 cm., spot 14-16 cm.
Mould enzyme	±1 cm., 3·5-10·5 cm., 10·5-12 cm.

(*b*) Sucrases

Acetone 40 per cent.; buffer: 0.1M phthalate, pH 5.7

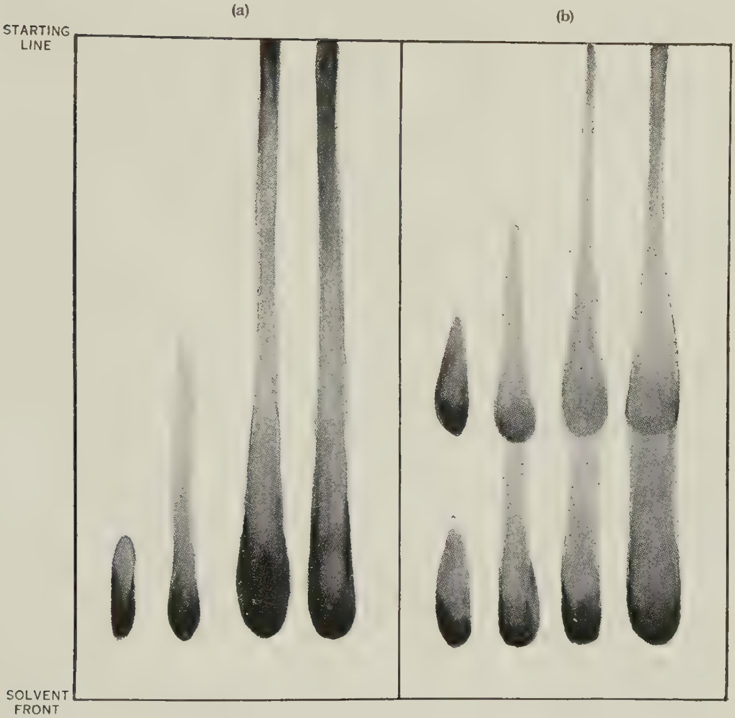
Taka diastase	Single spot 15-17·5
“Diastase”	Single spot 15·5-17·5
Invertase (liquid)	Spot ±0·5 cm., trail, streak 9·5-17·5 cm., spot 18-20 cm.
Invertase (crystalline)	Spot ±0·5 cm., streak 0·5-18 cm., spot 19-20 cm.
Mould enzyme	0-2, 3·5-9, Trail, 13·5-16, 16-19, 19-22.

(*c*) β-Glucosidases

Acetone 65 per cent.; buffer: 0.01M citrate-phosphate, pH 6.0

“Cellulase”	Streak 0·5-5 cm.; streak 8·5-11·5 cm.
“Emulsin”	Spot ±1 cm.
“Hemicellulase”	Streak 1-7 cm.; spot 11-13 cm.
Luizym	Spot 10-12 cm.
Malt β-glucosidase (lab. preparation)	Streak 0·5-4·5 cm.
Oat β-glucosidase (lab. preparation)	Spot ±1, spot 10-11 cm.
Apricot emulsin (lab. preparation)	Streak −0·5-4 cm.
Almond emulsin (lab. preparation)	Streak −0·5-7 cm.; spot 9-11 cm.
Mould enzyme	Streak −0·5-6 cm., spot 10-12 cm.

PAPER CHROMATOGRAPHY OF ENZYMES



Effect of increasing concentration of enzyme in the applied spot on the distribution of the enzyme after paper chromatography: (*a*) enzyme with a single component; (*b*) enzyme with two components. Enzyme concentrations increasing from left to right.

Table 7 (*Continued*)

(*d*) Salicinases

Acetone 50 per cent.; buffer: 0.01M citrate-phosphate, pH 4.0

Enzyme Source	Appearance After Solvent Front has Travelled 20 cm.
"Cellulase"	Long trail from origin to spot 14-17·5 cm.
"Emulsin"	Spot ±0·5 cm.
"Hemicellulase"	Spot ±1 cm.; spot 6-8·5 cm.; spot 12·5-15·5 cm.
Luizym	Spot 14·5-17 cm.
Malt β-glucosidase	Streak—1-2; streak 5-12·5 cm.
Oat β-glucosidase	Spot 11-13·5 cm.
Apricot emulsin	Spot ±1 cm.; spot 2·5-5·5 cm.; spot 9·5-13 cm.
Almond emulsin	Streak 0·5-12·5 cm.
Mould enzyme	Spot 7·5-10·5; spot 13·5-15·5

(*b*) *The Nature of Multiple Components*

Almost all applications of the techniques of filter-paper chromatography and filter-paper electrophoresis to the study of naturally occurring enzymes have so far agreed in finding that many of the enzymes are multi-component systems. These components may be pre-existent separate enzymes or artifacts of the separation process. Gillespie, Jermyn, and Woods (1952) and Jermyn (1952*b*) have shown that the components present in *A. oryzae* β-glucosidase and horse-radish peroxidase as revealed by paper chromatography cannot be artifacts and Gillespie and Woods (1953) have shown that it is possible to separate the multiple components revealed by paper electrophoresis in a number of *A. oryzae* enzymes.

Table 8

SUMMARY OF THE EFFECT OF VARIOUS FACTORS ON THE PAPER CHROMATOGRAPHY OF THE *A. ORYZAE* ENZYMES

Factor	General Effect Upon			
	Distance of Travel of Enzyme Spot	Definition of Spot	Inactivation of Enzyme	Trailing of Enzyme Spots
Concentration of enzyme spot	—	—	—	++
Nature of buffering ion	Feeble	+	+	+
Concentration of buffering ion	—	++	—	—
Nature of organic component	Feeble	+	++	+
Concentration of organic component	++	+	+	Feeble
pH	++	—	+	—

Giri *et al.* (1952) believe that the two alkaline phosphatases of acetone-treated kidney are quite independent enzymes. Cabib (1951) observed two zones of invertase activity without being able to show that they correspond to enzymes of different properties. Reid (1950, 1951) observed multiple zones of enzyme activity but could also advance no evidence as to whether he was dealing with multiple enzyme systems.

The following criteria are suggested for establishing that observed components are not artifacts. They must (i) be mobile under some conditions, (ii) appear independently of other proteins present as "impurities," (iii) show no signs of being interconvertible, and should preferably (iv) have distinguishably different properties and (v) be separable by some process of fractionation. In terms of these criteria only the two *p*-nitrophenyl-β-glucosidases (Jermyn 1952*b*, 1952*c*) and two of the proteases (Woods and Gillespie 1953) of *A. oryzae* have been shown to have an independent existence by using these techniques.

Where multiple components exist in one batch of the enzyme material and not in another, it also seems probable that they must have an independent existence. This is true for the *A. oryzae* phosphatases. After development using pH 5.0 acetate buffer and 40 per cent. acetone only a single phosphatase (pH optimum *c.* 5.0, but sufficiently active at pH 8.0 to be an "alkaline phosphatase") is visible in the batch used in this series of experiments, where three were visible in the batch used by Gillespie, Jermyn, and Woods (1952). Of these three, the most mobile corresponds exactly with the single acid phosphatase spot detected in the present study.

How far the components observed by filter paper electrophoresis and chromatography are identical has still to be decided. The preliminary results of Gillespie, Jermyn, and Woods (1952) have now been sufficiently refined for trustworthy comparison of the two methods to be made. There is agreement on the number of proteases (two) and disagreement on the number of esterases (two by electrophoresis and three by chromatography) and *p*-nitrophenyl-β-glucosidases (one by electrophoresis and two by chromatography). In other cases, such as amylase and salicinase, there is agreement on the multi-component nature of the enzyme, but the evidence from chromatographic experiments is not sufficiently precise to say whether there is agreement on the number of these components. It is evident that enzyme proteins with similar properties as judged by filter-paper electrophoresis may have dissimilar properties when judged by chromatography. The reverse case has not so far been demonstrated with certainty, but the six amylases that appear on electrophoresis have not been found in the chromatography experiments.

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FEEDING BEHAVIOUR OF THE APHIDS *MYZUS PERSICAE* AND *BREVICORYNE BRASSICAE*, STUDIED WITH RADIOPHOSPHORUS

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Summary

The volume of material ingested and excreted by the aphids *Myzus persicae* and *Brevicoryne brassicae* was measured by feeding them on leaves or solutions containing radiophosphorus. No uptake could be measured within the first few minutes of penetration by the stylets. *Myzus* ingested approximately 0.07 mg. plant material in 1 hr. *Brevicoryne* ingested less than one-twentieth of that amount in the same period. *Myzus* ingested more from the lower than from the upper surface of the leaf. No artificial diet was found that was ingested by *Myzus* as readily as leaf tissue.

Both species reinjected a fraction of 1 per cent. of the amount ingested during the subsequent feed. No evidence was obtained for the occurrence of regurgitation from the midgut.

Periods of starvation from 30 min. to 4 hr. did not affect the amount subsequently ingested by *Myzus* during a 30-min. feeding period.

The volume of liquid mechanically transported on the stylets of *Myzus* approximated 2×10^{-7} ml.

The relation of these observations to hypotheses of the transmission of viruses by aphids is briefly discussed.

I. Introduction

It is over 30 years since aphids were first incriminated as vectors of virus diseases of plants (Allard 1917). However, in no case has the mechanism of transmission been definitely established (Bawden 1950), although several hypotheses concerning this mechanism have been advanced (see for example Doolittle and Walker 1928; Watson 1936, 1946; Smith and Lea 1946; Bradley 1952). It is clear that further data are needed concerning details of the feeding behaviour of aphids to aid evaluation of the hypotheses that have been put forward.

Hamilton (1935) has already reported results on the feeding of *Myzus persicae*, using polonium as a tracer, but advances in the knowledge of isotopes since that time have made it desirable that some of her experiments be repeated. The results of a study of some aspects of feeding and excretion by the green peach aphid *Myzus persicae* (Sulz.) and the cabbage aphid *Brevicoryne brassicae* (L.) are presented in this paper.

II. Methods

Feeding was studied by permitting the insects to ingest radiophosphorus. This was obtained as $Na_2H^{32}PO_4$ or as $NaH_2{}^{32}PO_4$ from the British Atomic Energy Establishment through the Tracer Elements Investigations Section,

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C.S.I.R.O. The isotope was incorporated in sucrose solutions or into plant tissues by permitting the petiole of an isolated leaf of Chinese cabbage (*Brassica chinensis* L.) to stand in Knop's solution containing the isotope until a measured volume of solution had been absorbed by the leaf. Most of the equipment and techniques were the same as described by Day and McKinnon (1951). All data were corrected for background and when necessary, for decay. The controls and measures to eliminate contamination were satisfactory for each test. The work was greatly facilitated by the use of the plastic material "Plas-B-Loon" used by Day and McKinnon (1951). From this material very thin transparent membranes (hereinafter called "plastic membranes") were made. These were easily penetrated by the stylets of the aphids, but no diffusion of phosphate solutions occurred through the membranes even when highly active (0.1 ml. of solution containing 0.5 mc./ml.) sources of ^{32}P were used.

TABLE 1

INGESTION OF ^{32}P BY *M. PERSICAE* FROM LEAF OF CHINESE CABBAGE

Five insects per test, feeding time 1 hr.

Leaf No.	Position on the Leaf	Ingested ^{32}P (counts/min.)
1	Upper surface	106
	Underside	994
2	Upper surface	182
	Underside	2300

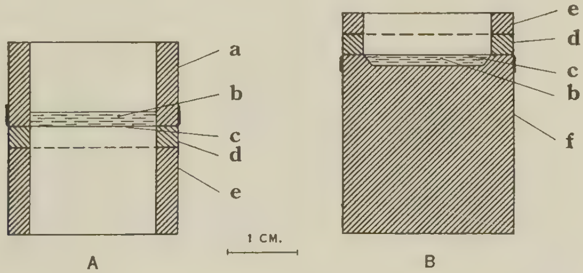

Fig. 1.—Arrangement for feeding aphids on artificial diets. *A*, *M. persicae*; *B*, *B. brassicae*. *a*, Plastic ring; *b*, feeding solution; *c*, plastic membrane; *d*, separating plastic ring; *e*, container for aphids; *f*, plastic block.

Apterae of both species of aphids starved for 30 min. were used in all experiments unless the contrary is stated.

III. Observations

(*a*) *Ingestion*

An examination was made of the amount of plant juices and of sucrose solutions ingested by *Myzus* and *Brevicoryne*. First, an experiment was performed with *Myzus* to determine whether this species ingests more when feeding from the upper or lower surface of the leaf. The data (Table 1) show that a greater amount was ingested when the insects fed on the underside of the leaf. Two factors may be involved, one resulting from differences in ^{32}P content of different leaf tissues, the other from the preferred orientation of the insects when feeding. The second alternative was shown to be concerned during artificial feeding, for *Myzus* ingested sucrose through a plastic membrane only when it was presented in the apparatus illustrated in Figure 1*A*. This arrangement was therefore used in subsequent experiments with *Myzus*. *Brevicoryne*, however, was unable to maintain its position on the plastic membrane under these conditions, and was therefore fed on sucrose solutions as shown in Figure 1*B*.

Table 2

WEIGHT OF PLANT MATERIAL INGESTED BY *M. PERSICAE*

Feeding Time	No. Insects (*a*)	Ingested ^{32}P (counts/min.) (*b*)	^{32}P Content in 1 mg. Leaf (counts/min.) (*c*)	Wt. of Material Ingested by One Insect (mg.) $\frac{(b)}{(a)\times(c)}$	Mean Wt. of Material Ingested by One Insect (mg.)
10 Min.	10	64	$1{\cdot}9\times10^4$	0·0003	0·0002
10 Min.	10	21	$1{\cdot}9\times10^4$	0·0001	
15 Min.	10	357	$1{\cdot}9\times10^4$	0·0019	0·0027
15 Min.	10	650	$1{\cdot}9\times10^4$	0·0034	
20 Min.	10	301	$1{\cdot}9\times10^4$	0·0016	0·0023
20 Min.	10	544	$1{\cdot}9\times10^4$	0·0029	
30 Min.	5	760	$1{\cdot}9\times10^4$	0·0080	0·0078
30 Min.	5	717	$1{\cdot}9\times10^4$	0·0075	
1 Hr.	5	6520	$1{\cdot}9\times10^4$	0·0686	0·0687
1 Hr.	5	6540	$1{\cdot}9\times10^4$	0·0688	
2 Hr.	5	8860	$1{\cdot}9\times10^4$	0·0934	0·0898
2 Hr.	5	8180	$1{\cdot}9\times10^4$	0·0862	
4 Hr.	10	12380	$6{\cdot}9\times10^3$	0·1795	0·1795
6 Hr.	10	19300	$6{\cdot}9\times10^3$	0·2795	0·2795

The weight of plant material, excluding the amount excreted, ingested by *Myzus* is shown in Table 2, and the comparable data for *Brevicoryne* in Table 3. It will be observed that the rate of uptake by the green peach aphid is many times greater than the rate of uptake by the cabbage aphid. In spite of the high concentrations of isotope employed (0.1 mc. in approximately 5 g. leaf) no evidence of uptake was measurable before 5 min., although extrapolation of data obtained after 15, 30, and 60 min. feeding indicates that, had it been linear with time, uptake should have been measurable. It is concluded that a measurable amount of ingestion does not occur during the initial stages of stylet penetration. However, during punctures of short duration the behaviour of the insect suggests that it can distinguish, presumably by taste, the tissues through which its stylets are penetrating. Gustatory organs appear to be absent from the stylets, but occur in the functional mouth just anterior to the pharynx. It seems likely that sufficient solution is ingested to stimulate these organs.

Table 3

WEIGHT OF PLANT MATERIAL INGESTED BY *B. BRASSICAE*

Feeding Time (hr.)	No. Insects (a)	Ingested ^{32}P (counts/min.) (b)	^{32}P Content in 1 mg. Leaf (counts/min.) (c)	Wt. of Material Ingested per Insect (mg.) $\frac{(b)}{(a)\times(c)}$	Mean Wt. of Material Ingested per Insect (mg.)
1	10	248	27600	0·0009	0·0027
1	30	3100	27600	0·0037	
1	30	2800	27600	0·0034	
2	10	2580	27600	0·0094	
2	30	15500	27600	0·0187	0·0166
2	30	17950	27600	0·0217	
4	10	3790	27600	0·0137	
4	30	17530	27600	0·0212	0·0275
4	30	39500	27600	0·0477	
6	5	18800	27600	0·136	
6	30	118000	27600	0·143	0·1310
6	30	94500	27600	0·114	

Table 4

VOLUME OF 10 PER CENT. SUCROSE SOLUTION INGESTED BY *M. PERSICAE*

Feeding Time	No. Insects (a)	Ingested ^{32}P (counts/min.) (b)	^{32}P Content in 1 cu. mm. Sucrose (counts/min.) (c)	Volume of Sucrose Ingested per Insect (cu. mm.) $\frac{(b)}{(a)\times(c)}$
45 Min.	40	303	3860	0·002
16 Hr.	20	920	2890	0·016

The volume of 10 per cent. sucrose solution ingested by *Myzus*, excluding the amount excreted, is shown in Table 4, and the comparable data for *Brevi-*

coryne in Table 5. *Brevicoryne* ingested sucrose almost as readily as it ingested plant material, but *Myzus* ingested sucrose very poorly. This is partly accounted for by a reluctance by *Myzus* to penetrate the plastic membrane, but other factors are also involved. Attempts to increase the acceptability of the artificial diet by the addition of several sugars, proteins, protein hydrolysate, asparagine, or mixtures of several amino acids were unsuccessful. Similarly, an experiment to determine whether *Myzus* showed pH preferences was made by feeding the insects on artificial diets, buffered by dilute phosphate buffers of pH 5.3-8.0. The results showed low uptake from all solutions but no difference between them. It was concluded that, until a more acceptable artificial diet was available for *Myzus*, the experiments using artificial media for the species were of doubtful value.

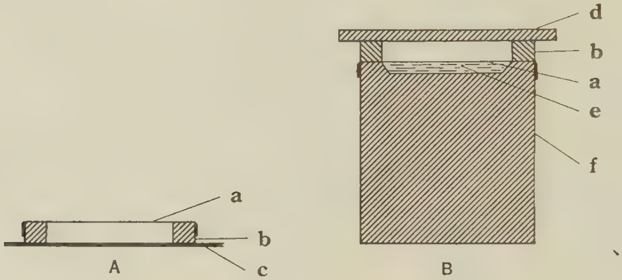

Fig. 2.—Arrangement for studying excretion by aphids. *A*, *M. persicae*; *B*, *B. brassicae*. *a*, Plastic membrane; *b*, plastic ring; *c*, leaf on which aphids were fed; *d*, glass cover; *e*, feeding solution free of radioactivity; *f*, plastic block.

Table 5

VOLUME OF 10 PER CENT. SUCROSE SOLUTION INGESTED BY *B. BRASSICAE*

Feeding Time (hr.)	No. Insects (*a*)	Ingested ^{32}P (counts/min.) (*b*)	^{32}P Content in 1 cu. mm. Sucrose (counts/min.) (*c*)	Volume of Sucrose Ingested per Insect (cu. mm.) $\frac{(b)}{(a)\times(c)}$
1	10	273	11800	0·002
2	10	1370	11800	0·012
3	10	130	11800	0·001
4	10	2970	11800	0·025
24	5	1480	11800	0·025

(*b*) *Excretion*

Excretion of isotope was measured by estimating the activity remaining in small containers after the removal of the insects. Excretion thus includes material originating from the cornicles as well as that of alimentary origin, but in these short feeding periods could not have included any reinjected material (see following section). This procedure may be expected to give low values for *Brevicoryne*, for some of the excreta of this insect normally remain on the insect. The arrangement of apparatus used to obviate contamination in this experiment is illustrated in Figure 2. The results of experiments to determine the percentage of ingested material excreted are given in Tables 6 and 7 for *Myzus* and *Brevicoryne* respectively. The variability of the results, especially where no excretion was recorded, indicates the discontinuous nature of the process.

TABLE 6

EXCRETION OF ^{32}P BY *M. PERSICAE* DURING FEEDING ON CHINESE CABBAGE LEAF

In each test 20 insects exposed on leaf containing ^{32}P for 3 hr.

^{32}P Ingested by Insects (counts/min.)	Time of Exposure to Radioactive Leaf (hr.)	^{32}P Content on Leaf (counts/min.)	^{32}P Content in Container (counts/min.)	^{32}P Excreted (% of amount ingested)
8940	½	7	232	2·7
7960	½	8	51	0·7
8120	½	0	168	2·1
9140	1	0	491	5·4
9940	1	0	379	3·8
5630	2	58	467	9·3
6290	2	149	844	17·4

TABLE 7

EXCRETION OF ^{32}P BY *B. BRASSICAE* DURING FEEDING ON 10 PER CENT. SUCROSE SOLUTION

Thirty insects used in each test

Time of Exposure to ^{32}P Leaf (hr.)	Ingested ^{32}P (counts/min.)	Time of Exposure to Sucrose (hr.)	Excreted ^{32}P (counts/min.)	Excreted ^{32}P (% of amount ingested)
1	3100	1	0	0
1	2800	1	189	6·8
2	15400	2	0	0
2	17800	2	90	0·5
4	17300	2	27	0·2
4	39000	2	646	1·7
6	112700	17	5300	4·7
6	89900	17	4020	4·5

Broadbent (1951) has reported that *Myzus* does not excrete on the leaf upon which it is feeding. This is frequently so, but it will be noted in Table 6 that sometimes substantial amounts of excreted phosphorus were detected on the leaf upon which the insects were feeding.

(*c*) *Reinjection of Ingested Radiophosphorus*

Using the data obtained on ingestion and excretion a series of experiments was designed to determine whether reinjection of ingested ^{32}P occurred. Precautions were taken to ensure against contamination from excreta and other sources. The liquid (*e*) in Figure 2*B* was removed quantitatively and its activity measured. The data in Tables 8 and 9 demonstrate that ejection of ingested ^{32}P by both species does occur, but that the amount is smaller than the 6.9 per cent. of ingested material reported by Hamilton (1935).

Table 8

EJECTION OF ^{32}P BY *M. PERSICAE* DURING FEEDING

No. Insects	Time of Exposure to ^{32}P Leaf (hr.)	Ingested ^{32}P (counts/min.)	Time of Exposure to Sucrose (hr.)	Excreted ^{32}P (counts/min.)	Excreted ^{32}P (% of amount ingested)
40	17	46600	24	66	0·14
40	17	46600	24	70	0·15
30	17	35000	24	71	0·20
30	17	35000	24	0	0·0
30	17	35000	24	51	0·15
30	23	97000	22*	11	0·01
30	23	59000	22*	8	0·01
		Mean percentage ejection for insects fed on sucrose solution			0·13

* Insects were fed through plastic membrane on non-radioactive leaf.

There are three routes by which this ejected ^{32}P could have reached the second feeding medium. Either the isotope was taken into the midgut, absorbed into the haemocoele, and reinjected with the saliva; or it was regurgitated directly from the midgut; or it reached the feeding medium by contaminating the stylets. The absorption of phosphate occurs very readily; an attempt was made to determine by radio-autographs whether the isotope was present in the *Myzus* salivary glands at various times after feeding. At all times studied, from 15 min. to 24 hr., the isotope was concentrated in the midgut and developing embryos, but it was also present throughout the body and in the salivary glands. In view of the long feeding times (17-24 hr.) required before reinjected ^{32}P could be detected, it seems more likely that it was reinjected in the saliva than by the other routes.

(*d*) *Effects of Duration of Starvation on Quantity of Material Ingested*

It has been confirmed by several authors following the observations of Watson (1938) that aphids transmit certain viruses more efficiently after a brief period of starvation. It has generally been assumed that the insects ingest more efficiently following starvation. It was, therefore, of interest to determine whether the quantity ingested differed following periods of starvation previously known to influence the efficiency of virus transmission. Groups of 20 *Myzus* were starved for periods from 30 min. to 4 hr. and were then permitted to feed for 30 min. on a leaf of Chinese cabbage containing ^{32}P. The amount ingested in two such experiments is shown in Table 10. Although variable, the data suggest that starved aphids ingest approximately the same amount as do unstarved aphids.

TABLE 9

EJECTION OF ^{32}P BY *B. BRASSICAE* DURING FEEDING

No. Insects	Time of Exposure to ^{32}P Leaf (hr.)	Ingested ^{32}P (counts/min.)	Time of Exposure to Sucrose Free of ^{32}P (hr.)	Excreted ^{32}P (counts/min.)	Excreted ^{32}P (% of amount ingested)
30	6	112700	17	637	0·57
30	6	89900	17	369	0·41
				Mean	0·49

TABLE 10

EFFECT OF STARVATION ON INTAKE OF FOOD BY *M. PERSICAE*

Twenty insects fed on radioactive leaf of Chinese cabbage for ½ hr.

Experiment No.	Time of Starvation (hr.)	Intake of ^{32}P (counts/min.)
1	No starvation	358
	½	272
	1	315
	2	294
	4	985
2	No starvation	628
	½	825
	1	301
	2	782
	2	700
	4	668
	4	856

(*e*) *Amount of Material Carried Mechanically on Feeding Stylets*

Certain workers on the mechanism of transmission of non-persistent viruses have suggested that the pathogens are carried mechanically on the mouth-parts. It was therefore of interest to determine the quantity of material that could be so carried. Stylets were dissected from *M. persicae* alatae and apterae. Approximately half the length of the stylets was immediately immersed for 10 sec. in droplets of water containing a known amount of isotope. The volume of liquid remaining on the stylets could then be determined (Table 11). Occasionally surface tension prevented the stylets from penetrating or caused the solution to contaminate the stylets over their entire length. Such stylets were discarded.

TABLE 11
VOLUME OF ^{32}P SOLUTION CARRIED MECHANICALLY ON FEEDING STYLET OF *M. PERSICAE*

Freshly Dissected Stylet Immersed in ^{32}P Solution*		Stylet Dissected and Dried in Air 30 Min., Then Immersed in ^{32}P Solution†		Dissected and Dried-off Stylet Immersed in ^{32}P Solution + Wetting Agent†	
^{32}P Carried on Stylet (counts/min.)	Volume Carried on Stylet (ml.)	^{32}P Carried on Stylet (counts/min.)	Volume Carried on Stylet (ml.)	^{32}P Carried on Stylet (counts/min.)	Volume Carried on Stylet (ml.)
14	$4 \cdot 0 \times 10^{-8}$	3	—	0	—
35	$1 \cdot 0 \times 10^{-7}$	47	$1 \cdot 8 \times 10^{-7}$	2	—
4	—	20	$7 \cdot 7 \times 10^{-8}$	4	—
29	$8 \cdot 3 \times 10^{-8}$	88	$3 \cdot 4 \times 10^{-7}$	16	$6 \cdot 2 \times 10^{-8}$
224	$6 \cdot 4 \times 10^{-7}$	81	$3 \cdot 1 \times 10^{-7}$	2	—

* 1.0×10^{-6} ml. ^{32}P solution = 350 counts/min.
† 1.0×10^{-6} ml. ^{32}P solution = 260 counts/min.

Some stylets were permitted to dry out for approximately 30 min. in air before immersing them. This did not alter the volume of liquid picked up. Nor was there any difference between the results with alatae and apterae. The incorporation of a small amount of wetting agent ("Santomerse D," Monsanto Chemical Co.) in the water permitted the stylets to penetrate the droplet readily, but reduced the amount picked up almost to the limit of volume measurable. This limit approximated 4.0×10^{-8} ml. with the isotope available. It was observed that the stylets separate and the mandibles curve away from the maxillae in a solution containing wetting agent, whereas they remain fasciculated when introduced into water alone.

IV. DISCUSSION

The results presented may be compared with those obtained by Hamilton (1935) on *Myzus persicae* and by Day and McKinnon (1951) on the leafhopper

Orosius argentatus. Plant-feeding Hemiptera generally ingest large volumes relative to body weight. Under the most favourable conditions *Orosius* ingested about 15 per cent. of its weight per hour. Hamilton's (1935) data show that the mean weight of a single *Myzus* approximates 0.54 mg. The *Myzus* used in the present study were smaller and weighed approximately 0.2 mg. This species therefore ingests roughly 35 per cent. of its weight in 1 hr. This figure is greatly in excess of that reported by Hamilton; but this is partly accounted for by the observation that *Myzus* ingests so much less of a sugar solution than it does when feeding upon a leaf.

Whereas Hamilton's data on ingestion are too low, her results on reinjection are higher than those reported above. This is undoubtedly due to the fact that some excreted isotope was absorbed by the plant. Broadbent (1951) has shown that the excreta of *Myzus* does not ordinarily lodge on the surface on which the insect is feeding, but the data reported here prove that occasionally substantial amounts of excreta may appear on the leaf.

The amount excreted by the aphids is considerably less than that excreted by *Orosius.*

Certain of the data obtained have a bearing on hypotheses relating to the mechanism whereby aphids transmit phytopathogenic viruses. Thus non-persistent viruses may be transmitted within a 2-min. feeding period. Our data indicate that no ingestion occurs in so short a period after penetration by the stylets. Short periods of starvation increase the food uptake of *Orosius* but do not appear to affect *Myzus.* It is possible that the 30-min. feeding period necessary to record amounts of ingested material sufficient for accurate measurement may vitiate the effects of starvation, but it seems likely that the effect of starvation on the ability of aphids to transmit non-persistent viruses may be due to factors other than the amount ingested.

The reliability of the data on the quantity of material reinjected by *Myzus* would be improved if that species fed better on the artificial medium. The results for *Brevicoryne* are higher and more reliable, but the volume reinjected is still very small. A low percentage of ^{32}P in salivary glands was also reported by Hahn, Haas, and Wilcox (1950) for the mosquito *Aedes aegypti* and by Flemion, Weed, and Miller (1951) for the bug *Lygus oblineatus.* Reinjection by this bug took place through salivary secretions (Flemion, Miller, and Weed 1952). As with *Myzus* there was no evidence for regurgitation from the midgut.

It is apparent that the amount of virus transmitted by an aphid to a susceptible host is extremely small, whether it be transported through the saliva or by contamination of the mouth-parts. Infection by mechanical inoculation is rarely successful even for the most infectious viruses at dilutions of cell sap greater than about 10^5. The ability of the aphid to place the virus in a manner in which it can infect the host is therefore far greater than can be effected by any of the methods of mechanical inoculation so far devised.

V. ACKNOWLEDGMENTS

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STUDIES ON THE DIGESTION OF WOOL BY INSECTS

VIII. THE SIGNIFICANCE OF CERTAIN EXCRETORY PRODUCTS OF THE CLOTHES MOTH, *TINEOLA BISSELLIELLA*, AND THE CARPET BEETLE, *ATTAGENUS PICEUS*

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Summary

The excreta of clothes moth larvae (*Tineola*) bred on a standard undyed woollen fabric, the black carpet beetle (*Attagenus*) living on wool, and the potato moth (*Gnorimoschema*) feeding on potato tubers, have been examined. Water-soluble nitrogen constitutes most of the total nitrogen in both *Tineola* and *Attagenus* excreta. The fraction of this water-soluble nitrogen contributed by uric acid or its salts is high in *Tineola*, but considerably lower in *Attagenus*. Up to 3 per cent. urea and an appreciable quantity of ammonia are also present. Small quantities of urea (0.14 per cent.) were found in the dissected midguts of *Tineola* larvae feeding on wool, but none was found in several non-keratin-feeding insects. The concentration present in the *Tineola* midgut is too small to be of significance in the digestion of wool through any direct denaturing action on keratin.

In *Tineola* the sulphur of wool is not excreted to any large extent as inorganic sulphate or as sulphur dioxide but mainly as cystine. *Attagenus* excretes almost twice as much cystine as *Tineola* on a similar diet. Lanthionine was not found in *Tineola* excreta. *Tineola* larvae fed wool treated with nickel salts excrete black faecal pellets having a much reduced cystine content. It is suggested that the cystine is broken down (probably enzymically) to yield sulphur for the formation of nickel sulphide.

I. Introduction

An examination of the end products of digestion in keratin-feeding insects should provide valuable data for the study of the digestive processes in these insects. Although some analyses do appear in the literature, a number of questions, some assuming greater importance in the light of recent work, remain unanswered. The fate of the keratin sulphur, after digestion by insects, is of considerable interest as the presence of sulphur is essential to the structure and stability of the keratin molecule (Geiger and Harris 1942). Babcock (1912) suggested that sulphur dioxide might be a metabolic product of clothes moth larvae feeding on wool, while Schultz (1925) concluded that the sulphur of keratin is excreted essentially as sulphate. The source of the sulphur required for the formation of metallic sulphides in the *Tineola* gut (Waterhouse 1952*a*) is also of interest. Wool is chemically changed with formation of lanthionine after treatment with alkalis (Cuthbertson and Phillips 1945) and recent work of Blackburn (1950) shows that wool thus treated becomes more easily digested by papain-bisulphite mixture than is normal wool. It was suggested that diges-

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tion of wool in the alkaline *Tineola* gut (about pH 10 (Linderstrøm-Lang and Duspiva 1936; Waterhouse 1952*b*)) might depend to a large extent on this reaction. Linderstrøm-Lang and Duspiva (1936) drew attention to the presence of urea in the gut of *Tineola* and inferred that urea might be involved in the digestion of wool in the larvae through some denaturation of the keratin. No quantitative data, however, were presented with which to determine the significance of the amounts of urea found in the larval gut.

II. Methods

(*a*) *Insect Material*

Larval excreta from cultures of *Tineola bisselliella* (Humm.) feeding on white woollen fabric, were obtained by sieving and winnowing with a small air jet until free from loose wool fragments. Samples of excreta from (i) *Tineola* which had infested a large number of dead beetles (*Aphodius howitti* Hope), (ii) from the black carpet beetle (*Attagenus piceus* (Oliv.)) on wool, and (iii) from potato moth (*Gnorimoschema operculella* (Zell.)) on potato tubers, were collected in a similar manner. Insect material required for the urea and ammonia estimations in the gut was drawn from laboratory cultures of *Tineola bisselliella, Locusta migratoria* L., *Agrotis infusa* (Boisd.), and *Tribolium confusum* Duv., and from field-collected *Aphodius* and melalonthid and dynastid larvae.

(*b*) *Chemical Methods*

Dry matter content: dried to constant weight at 105°C. Water-insoluble matter: excreta heated with water on boiling water-bath for 1 hr., filtered, and dried to constant weight at 105°C. Ammonia and urea: urease method of Hawk, Oser, and Summerson (1947, p. 822). The xanthydrol method of Engel and Engel (1947) was used as a check on the urea figures. Nitrogen: micro-Kjeldahl. Amino nitrogen: micro-titrimetric ninhydrin method of Van Slyke, MacFadyen, and Hamilton (1941) modified by using Thunberg tubes instead of the recommended U-tubes and flasks, the barium hydroxide being held in the hollow stoppers. Creatine and creatinine: alkaline picrate method of Folin and Wu (1919) using tungstate filtrate. Creatine hydrolysed with HCl in an autoclave at 20 lb./sq. in. pressure for 20 min. Total and soluble sulphur: Micro-Carius digestion followed by precipitation of benzidine sulphate (Niederl *et al.* 1940). The precipitate was dissolved in hot N/50 NaOH and back-titrated with N/50 HCl, using neutral red as indicator. Sulphate sulphur: gravimetric as barium sulphate. Cystine: the colorimetric method of Shinohara (1935) using the reagent of Newton (1937). Total cystine was estimated on the hydrolysate obtained after refluxing for 4 hr. in 5N HCl. Sulphur dioxide: the method of Grant (1947) was applied. All figures for soluble constituents of the excreta were obtained on hot water extracts except amino nitrogen, sulphate, and cystine, in which dilute hydrochloric acid extracts were used.

III. Experimental and Results

The results of the analyses of excreta from the present work are presented in Table 1, together with figures from other work on clothes moths. Some analyses (Brown 1937) of the excreta of *Melanoplus bivittatus* are included for

Table 1

ANALYSES OF EXCRETA OF *TINEOLA* AND OTHER INSECTS

	Tineola bisselliella			*Tinea pellionella*			*Gnorimoschema operculella*	*Melanoplus bivittatus*	*Attagenus piceus*
	This Paper		Hollande and Cordebard (1926)	Babcock (1912) *	Schultz (1925)	Mosher (1941)	This Paper *	Brown (1937) *	This Paper *
	* (Wool)	* (Beetle)							
Dry matter	95·2	91·2	99			92·6	93·8	92·5	93·3
Water-insoluble	25	30	18		28·4	25	59	56·5	31
Ash	1·06	9·92	5·5			9·67	11·8		
Total Nitrogen	24·9	19·0	19·7	26·66		19·74	1·80	4·24	22·5
Soluble nitrogen	21·1	16·2			6·39		1·03		17·8
Purine bases nitrogen				0·13					
Amino nitrogen	1·2							0·32	
Uric acid	41	31	28	38·1		28·46	2·0	4·7	2·8
Ammonia	4·1	2·8	3·0	3·31		2·04	0·05	0·08	0·97
Urea	3·0	1·5	0·4	10·1		Trace	0·06	0·34	3·0
Creatinine	<0·1					Nil			
Creatine	<0·1			7·2		Trace		Nil	
Allantoin						Nil			<0·1
Total sulphur	4·5	0·95			†		0·28	1·0	
Soluble sulphur	3·1								
Soluble SO_4 sulphur†	0·24	Nil	Trace		0·76		Nil		
Ethereal sulphate sulphur	Nil				Nil				
Insoluble sulphur	1·4 (by diff.)				1·43				
Total cystine	10·0	1·4			Not found		Nil	Doubtful	
Soluble cystine	6·2								11·8
Soluble lanthionine	Nil								
Insoluble lanthionine	Nil								

* These figures are quoted on a dry weight basis.

† Schultz (1925) states that total sulphur by the Liebig method corresponded to the sulphate sulphur.

comparison. All results from the present work are quoted to the nearest significant figure..

The water-insoluble fraction of a sample of *Tineola* larval excreta was examined by microscope and appeared to consist entirely of small fragments of undigested or partly digested wool fibres. This insoluble fraction, together with a sample of the original woollen fabric, was analysed for cystine content.

TABLE 2

CYSTINE CONTENT OF WATER-INSOLUBLE MATERIAL FROM *TINEOLA* EXCRETA

Results expressed as percentage of dried material

	Cystine		
	1	2	Mean
Insoluble matter from *Tineola* excreta	12·1	12·8	12·5
Woollen fabric	10·5	10·9	10·7

A paper chromatographic examination using phenol-water and acetone-water mixtures, and specific reagents for sulphur compounds, including iodoplatinate (Winegard, Toennies, and Block 1948) failed to show the presence of lanthionine in *Tineola* excreta, either before or after hydrolysis. The possible excretion of sulphur dioxide by clothes moth larvae was checked by analysis of the atmosphere passing over a group of 300 *Tineola* larvae feeding on wool. The air was drawn over the larvae at the rate of 5 l. in 2 days and passed through an absorbent of dilute alkali. No sulphur dioxide was detectable (< 0.2 μg.).

TABLE 3

ANALYSES OF EXCRETA FROM *TINEOLA* LARVAE ON TREATED WOOL

All results reported as percentage of dried excreta

Treatment	Cystine (Soluble in dilute HCl)	Nitrogen (Soluble in dilute HCl)	Nitrogen (total)
Nickel-treated fabric	1·4	4·6	19·1
Untreated fabric	6·7	7·8	26·0

An examination was made of a sample of excreta from *Tineola* larvae fed on woollen fabric treated with nickel salts. The standard white fabric was used as in the other experiments but it contained about 25 per cent. of its original weight of nickel sulphate, and a control test was carried out with untreated fabric. The analytical method for cystine (Shinohara 1935) was shown to be unaffected by the presence of nickel sulphate or nickel sulphide, in the amounts expected. The excreta from the larvae on the treated fabric were almost black in comparison to the normally straw-coloured excreta. The results of the analyses are presented in Table 3, and are discussed later.

The amount of urea in tissues of *Tineola* larvae and of some other insects is reported in Table 4. Both the clothes moth and the grasshopper (*Locusta*) were divided up into appropriate tissues for the analyses. *Tineola* was divided into (*a*) the hind part of the body together with the hindgut, (*b*) the midgut, and (*c*) the remainder of the body including head, while the *Locusta* gut was separated into (*a*) crop, (*b*) caeca, and (*c*) hindgut.

TABLE 4

UREA AND AMMONIA CONTENT OF INSECT TISSUES

All results reported as percentage of fresh tissues

Insect Material	Urea	NH_3
Tineola, whole larvae	0·14	0·04
Tineola, hind (*a*)	0·16	0·20
Tineola, midgut (*b*)	0·14	0·05
Tineola, body (*c*)	0·05	0·03
Agrotis, larvae, entire gut	Nil	0·01
Locusta, crop (*a*)	Nil	0·02
Locusta, caeca (*b*)	Nil	0·06
Locusta, hindgut (*c*)	Nil	0·05
Aphodius, larvae, entire gut	Nil	0·04
Melalonthid larvae, entire gut	Nil	0·07
Dynastid larvae, entire gut	Nil	0·02
Tribolium, whole larvae	Nil	0·01

IV. DISCUSSION

The three common nitrogenous excretory products, uric acid, urea, and ammonia, are all present in *Tineola, Attagenus*, and *Gnorimoschema* excreta. The figures for the latter are quite low compared to those for *Tineola* and *Attagenus*, probably owing to the relatively low protein content of the food of the potato moth. After the ingestion by an insect of a high sulphur-containing diet such as keratin, it might be expected that ammonia would be required to assist in the excretion of sulphate (Wigglesworth 1931). A calculation from the figures in Table 1, however, shows that only about 0.1 per cent. ammonia would be required to combine with the small quantity of sulphate in *Tineola* excreta. Although the water-soluble nitrogen figures for both *Tineola* and *Attagenus* are of the same order (21.1 and 17.8 per cent. respectively), ammonia, urea, and uric acid can account for as much as 86 per cent. of soluble nitrogen from *Tineola* but only about 17 per cent. from *Attagenus*. The low uric acid figure is notable in *Attagenus* and it was therefore thought that nitrogen might be excreted in the form of allantoin as in certain other insects, e.g. *Lucilia* (Robinson 1935). However, no allantoin was found and no further attempts were made to establish the identity of the nitrogenous excretory products.

Linderstrøm-Lang and Duspiva (1936) suggested that reduction of wool in the insect gut might be due to the presence of a hydrogen-transferring enzyme such as is involved in the Schardinger reaction. It seemed possible, in view of the high uric acid excretion in *Tineola* (Table 1), that xanthine oxidase, an enzyme responsible for the production of uric acid, might be involved. While this may be true in *Tineola* it seems much less likely in *Attagenus*, which has a low uric acid excretion, although it digests wool readily. The 41 per cent. uric acid excreted by *Tineola* appears to be high, but it is not exceptionally so, as the dried urine of the non-keratin-feeding *Rhodnius* contains 64-84 per cent. (Wigglesworth 1932). The possibility still remains that dehydrogenase activity takes part in the digestive processes of some keratin-feeding insects.

Three per cent. of urea was found in both *Tineola* and *Attagenus* excreta when feeding on wool, and *Tineola* feeding on *Aphodius* remains excreted 1.5 per cent. On general grounds one would not expect urea to be a "favourable vehicle" for the excretion of nitrogen in these keratin-feeding insects because of the relatively dry diet (Wigglesworth 1931). However, it is clear that an appreciable amount of urea is a normal constituent of the excreta of these insects and that it is of metabolic origin, in contrast to that of *Rhodnius*, which is largely derived from the blood upon which the insects feed (Wigglesworth 1931).

Small quantities of urea were found in whole *Tineola* larvae (Table 4) and it was first thought that this could be accounted for by the urea content of the faecal pellets in the hindgut. However, on dissection, it was shown that the *Tineola* midgut contains 0.14 per cent. urea, while other species examined contained none, although the ammonia concentrations were comparable. The presence of this urea in *Tineola* gut might appear to be of significance in the digestion of wool by insects in view of the known effects of urea on proteins, e.g. the denaturation of proteins (Hopkins 1930) and the depolymerization of actin (Szent-Gyorgi and Joseph 1951). However, these authors show that high concentrations of urea (about 3-5M) are required for the effects to appear. Assuming that the gut contents occupy one-half the volume of the total gut, the urea concentration may become about 0.28 per cent. or about 0.05M in the contents. It is known that certain changes in the contents of the gut, such as the loss of birefringence of wool and other indications of breakdown, occur quite rapidly and in a small clearly defined part of the *Tineola* midgut (Day 1951*a*). If all the urea found in the *Tineola* gut were concentrated in the region of this noticeable activity, approximately one-tenth of the length of the gut, the maximum local concentration of urea would still only be about 0.5M, which is very much lower than that required for any denaturing action.

It is also interesting to note in view of the restricted air supply to the *Tineola* gut (Day 1951*b*) that the speed of induction of gels in concentrated albumin solutions by urea is increased under anaerobic conditions (Huggins, Tapley, and Jensen 1951). However, since these authors have shown that this gelation follows the formation of a lattice structure by a rearrangement of sulphydryl and disulphide groups, it would seem that the action of concentrated urea under these conditions would tend to preserve the three-dimensional molecular structure of keratin rather than disrupt it. Although the effect of dilute urea solutions on proteins under anaerobic conditions is not known, it is unlikely that the concentrations of urea in *Tineola* gut could contribute to the digestion of wool by any direct and independent action on the fibres. This is supported by Mercer's (1949) observation that urea solutions dissolve the "unstabilized lower levels" of the wool root but have no effect on hardened keratin. No analysis of *Attagenus* gut has been made for urea concentration but it can be expected to be similar to *Tineola* in view of the similar concentrations in the excreta.

When considering the combined effects of urea and enzyme systems it is notable that Steinhardt (1938), De Jaffe (1947), and others have found that pepsin, trypsin, and some other enzymes are not inhibited by up to 10 per cent. urea. Furthermore, Lennox (1952) has found that urea increases the digestion of wool by some enzymes in the presence of reducing agents. The gut of both *Tineola* and *Attagenus* are very reducing, but nothing is known of the effect of urea on the activity of the proteinases of these insects. However, the maximum local concentration of about 0.5M would seem to be of little significance when the rate of digestion of wool by the urea-activated papain only increases about one-third at this urea concentration (Lennox 1952).

The figure for total sulphur in clothes moth excreta (Table 1) confirms that of Titschack (1922) (4.03-4.62 per cent.). Schultz (1925) observed some crystalline material in the faecal pellets of *Tinea* which resembled cystine crystals, but concluded that the sulphur of keratin is excreted mainly as inorganic sulphate and that cystine was absent. However, Pradhan (1949) examined the excreta of *Anthrenus fasciatus* Herbst. and, on the grounds of crystal structure and solubility, expressed the opinion that cystine was present. It may be calculated from Table 1 that 55 per cent. of the total water-soluble sulphur excreted by *Tineola* is cystine sulphur, but only 8 per cent. is sulphate sulphur. Cystine is also excreted in high concentration (11.8 per cent.) by *Attagenus*. It is clear that cystine rather than sulphate is the important sulphur-containing excretory product in *Tineola* and *Attagenus* and probably also in the closely related genera *Tinea* and *Anthrenus*.

The presence of cystine in keratin-feeding insects is interesting in view of the paucity of information on the excretion of amino acids by insects in general (Wigglesworth 1950). Since the keratin ingested by clothes moth larvae and carpet beetles is digested under highly reducing conditions, one would expect the production in the gut of cysteine rather than cystine. As we have seen, the sulphur of this cysteine is excreted principally as cystine and very little appears as sulphate. This provides an interesting parallel to the work on vertebrate cystinuria (Brand, Cahill, and Harris 1935) which shows that ingested cysteine is excreted as cystine, whereas the sulphur of ingested cystine appears as sulphate through a different catabolic process.

The black carpet beetle (*Attagenus*) excretes almost twice as much cystine as *Tineola* when feeding on a similar diet (Table 1). This is of interest as Waterhouse (1952*c*) has found that *Attagenus*, in contrast to *Tineola*, does not form metallic sulphides in the gut when fed wool treated with metallic salts. It is notable that when *Tineola* excretes black faecal pellets after feeding on nickel-treated fabric, the cystine content is much lower than after feeding on normal fabric (Table 3). Any dilution of the cystine in this experiment by nickel compounds should be reflected in a drop in nitrogen content; however, this occurs to a much smaller extent than the drop in cystine content. These differences between the insects may be explained by the presence, in *Tineola,* of an enzyme that decomposes cysteine with the production of hydrogen sulphide, and its absence in *Attagenus.* Such an enzyme is cysteine desulphydrase as found in the liver of the rat and dog (Fromageot, Wookey, and Chaix 1941). One of the products of the action of this enzyme is pyruvic acid and this has been demonstrated, using 2-4-dinitrophenylhydrazine (Lu 1939), in both *Tineola* and *Attagenus* excreta. Preliminary tests *in vivo* (Waterhouse 1952*c*) have indicated that the clothes moth larva does contain an enzyme of this type and *in vitro* confirmation will be reported more fully in a later paper.

Table 2 shows that the water-insoluble fraction of *Tineola* excreta (undigested wool fragments) contains slightly more cystine than the original woollen fabric. This may be due to the differential digestion of wool fractions of different chemical composition, on passage through the insect gut. In support of this it has been shown that the scales of wool contain a higher proportion of proline (Lindley 1947) and cystine (Geiger 1944), and that they are less easily attacked by enzymes than the cortex (Hock, Ramsay, and Harris 1941; Reumuth 1946).

The proteinase of *Tineola* has no special characteristics that could explain the digestion of wool by the larvae (Powning, Day, and Irzykiewicz 1951) but it was thought that the highly alkaline nature of the larval gut could contribute in some way. The larva of *Tineola* has a midgut pH of about 10 (Linderstrøm-Lang and Duspiva 1936; Waterhouse 1952*b*), which is unusually high, although midgut alkalinity has been shown to be characteristic of the Lepidoptera whether carnivorous or nectar- or wax-feeding (Waterhouse 1949). A possible product of the action of alkali on wool is lanthionine (Cuthbertson and Phillips 1945) but none was found in a chromatographic examination of the water-soluble fraction of the excreta. Also, the excreted undigested wool fragments constituting the water-insoluble fraction (Table 2), contained more rather than less cystine than the woollen fabric upon which the insects had been feeding. It may be concluded that neither *Attagenus,* which has a midgut pH of about 7 (Waterhouse 1952*c*), nor *Tineola* depend solely upon gut alkalinity for the digestion of wool.

V. ACKNOWLEDGMENTS

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FURTHER STUDIES ON THE DIGESTION OF WOOL KERATIN BY PAPAIN-UREA: THE EFFECT OF ADDING COMPOUNDS THAT RUPTURE DISULPHIDE BONDS IN ALKALINE SOLUTION

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[*Manuscript received September* 2, 1952]

Summary

Whereas the optimum pH for digestion of wool by papain-bisulphite-urea is 7.0, a higher pH value is required for optimum digestion when bisulphite is replaced by sodium thioglycollate, cysteine hydrochloride, or potassium cyanide. Thioglycollate at pH 9.5 is superior to cysteine at the same pH or to bisulphite at pH 7.0 in promoting wool digestion by papain solutions containing urea. Cyanide is least effective. When wool is incubated in solutions of bisulphite, thioglycollate, cysteine, or cyanide in the absence of papain and urea, the extent of splitting of disulphide bonds runs parallel with the enzyme digestion observed in the presence of these reagents. The number of disulphide bonds split was measured by the iodosobenzoic acid method.

Wool digestion in a solution containing only fully active papain and urea is less than one-tenth of that observed when sodium bisulphite is also present. The cystine-free protein, collagen, is digested most rapidly in papain-thioglycollate-urea at acid pH values.

At all pH values the presence in the papain solution of both urea and a reagent rupturing disulphide bonds leads to greater wool digestion than either of these compounds used singly with papain. The combined action of sodium bisulphite and sodium thioglycollate in papain-urea solution increases wool digestion over a range of pH values.

I. Introduction

It was previously reported that the digestion of wool in papain-bisulphite solutions is increased by the addition of urea and reaches completion within a few hours at 50-70°C. (Lennox 1952). When the urea is replaced by structurally related compounds less marked effects on the digestion are observed except with thiourea. Replacement of the sodium bisulphite with sodium thioglycollate, cysteine hydrochloride, or potassium cyanide greatly reduces wool digestion at pH 7, but in alkaline solution, as will be shown in the present paper, these compounds increase digestion in papain solution both in the presence and absence of urea.

Marriott (1928), who was one of the earliest workers to study keratin digestion, reported that any substance capable of rupturing the disulphide linkage of cystine can function as an activator for the depilation of hides. Thus sodium sulphide and potassium cyanide in lime suspension caused "rapid rotting of hair," while sodium sulphite caused hair loosening. Partial solution of wool in

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0.5M sodium thioglycollate at approximately pH 12 and in 1M potassium cyanide containing 0.1N sodium hydroxide was similarly attributed by Goddard and Michaelis (1934) to the splitting of the disulphide bonds. No reference to the action of alkaline solutions of cysteine on wool has been found in the literature but this compound was shown by Mirsky and Anson (1935) to be capable of reducing the disulphide bonds in serum albumin although it was less effective for this purpose than thioglycollate. Cysteine has frequently been used to activate papain (Fruton and Bergmann 1940).

The simultaneous action of thioglycollate and proteolytic enzymes on wool has not been previously studied although Goddard and Michaelis (1935) showed that proteins prepared by extraction of wool with alkaline thioglycollate, cyanide, or sulphide and acidification of the extract were digested by trypsin and pepsin, and Routh and Lewis (1938) reported that the attack on the thioglycollate extractive "kerateine" was more rapid than that on finely powdered wool. Geiger *et al.* (1941) made similar observations using wool that had been reduced with alkaline thioglycollate or reduced and treated with methyl iodide to methylate the sulphydryl groups and prevent re-formation of the disulphide bonds. This product was almost completely digested by crystalline pepsin and crystalline chymotrypsin but was scarcely attacked by crystalline trypsin and was claimed to be completely resistant to crystalline papain.

II. MATERIALS AND METHODS

Wool of 64's merino quality was used. The weathered tip was removed from the wool staples before Soxhlet extraction with ethanol and repeated washing with distilled water to remove wax and suint. Analytical grade chemicals were used throughout and the papain was a high quality product from Ceylon. All pH adjustments were made with hydrochloric acid or sodium hydroxide using a glass electrode pH assembly.

Digestion was measured by incubating 0.5 g. wool in 15 ml. of the test solutions in stoppered glass tubes at 50°C. and washing, drying, and weighing the undigested residue.[*]

The sulphydryl sulphur in partially reduced wool was measured by an adaptation (see Ellis, Gillespie, and Lindley 1950) of the iodosobenzoic acid method of Hellerman and Chinard (1941). In the present investigation immersion of 0.02 g. wool for 2 hr. in 20 ml. 0.002M iodosobenzoic acid was necessary for complete reaction with the sulphydryl groups. Unreduced reagent was estimated by the addition of potassium iodide and titration of the liberated iodine with 0.002N thiosulphate. Concentrations of sulphydryl sulphur estimated by the iodosobenzoic acid method were higher and less variable than the values determined by the Shinohara method (Shinohara 1935).

[*] In one experiment 56's Corriedale wool was incubated in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea. After 4 hr. at 50°C. and at pH 6.0, 6.5, 7.0, and 7.5 the digestion was only one-half to three-quarters of that observed with standard fine merino wool under the same conditions.

A sheep pelt was used as a source of collagen. The wool had been removed by application of a lime-sulphide depilatory to the flesh side and the pelt was delimed, bated, pickled in sulphuric acid and sodium chloride, freed of acid by repeated washing with a solution containing sodium acetate and sodium chloride, then with water, and finally dehydrated with acetone and air-dried.

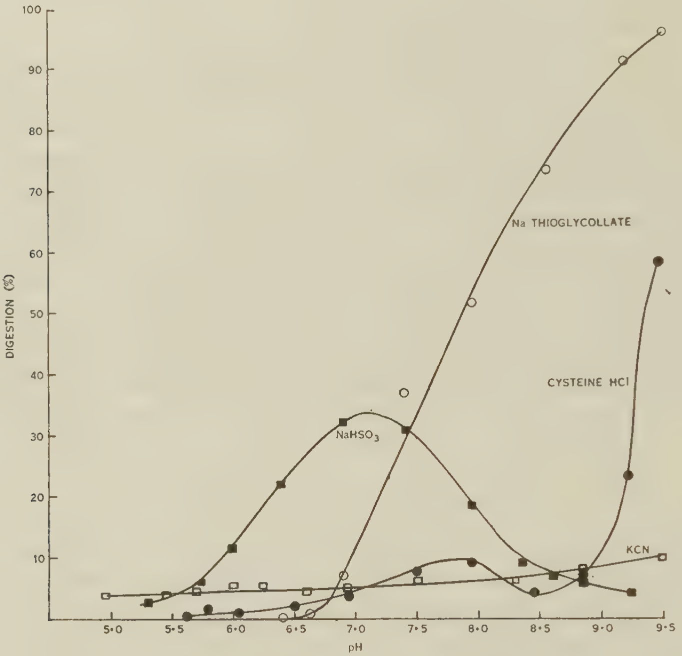

Fig. 1.—pH-wool keratin digestion in 1 per cent. papain, 3M urea solutions containing also various compounds that rupture disulphide bonds at 0.1M concentration. pH value measured after 0.5 hr. and digestion after 1.0 hr. incubation at 50°C.

III. Results

Figure 1 shows the effect of pH on the digestion of wool for 1 hr. at 50°C. in 1 per cent. papain, 3M urea solutions, containing in addition 0.1M sodium bisulphite, sodium thioglycollate, cysteine hydrochloride, or potassium cyanide. Sodium bisulphite produced maximum digestion at pH 7 while with the other reagents digestion increased as the solutions became more alkaline. Thioglycollate was the most effective agent under these conditions.

Figures 2, 3, 4, and 5 show the digestions obtained in similar solutions after 18 hr. incubation at 50°C. For comparison, curves showing the effect of omitting either urea or papain are also included. The pH values were measured 0.5 hr. after commencing the experiment. They were not separately determined for the papain-free and urea-free solutions but for purposes of graphing were assumed to change to the same values as those for the corresponding solutions containing all three components. The increase in digestion following the incorporation of urea in the solution containing papain and compounds rupturing disulphide bonds was greatest with bisulphite and least with cyanide. The digests containing cyanide at pH values above 8.5 were reddish brown in colour. After 18 hr. at 50°C., the thioglycollate series revealed a second digestion maximum at pH 7 which was not detected after 1 hr. at 50°C.

No significant digestion was observed after incubation for 18 hr. at 50°C. in 1 per cent. papain, 3M urea solutions containing 0.1M ascorbic acid as the reducing agent. When 0.1M sodium sulphide was used as the reducing agent in the papain-urea solutions, two maxima of approximately 40 per cent. digestion were observed at pH 7 and pH 9, but the results were variable and are therefore not reported.

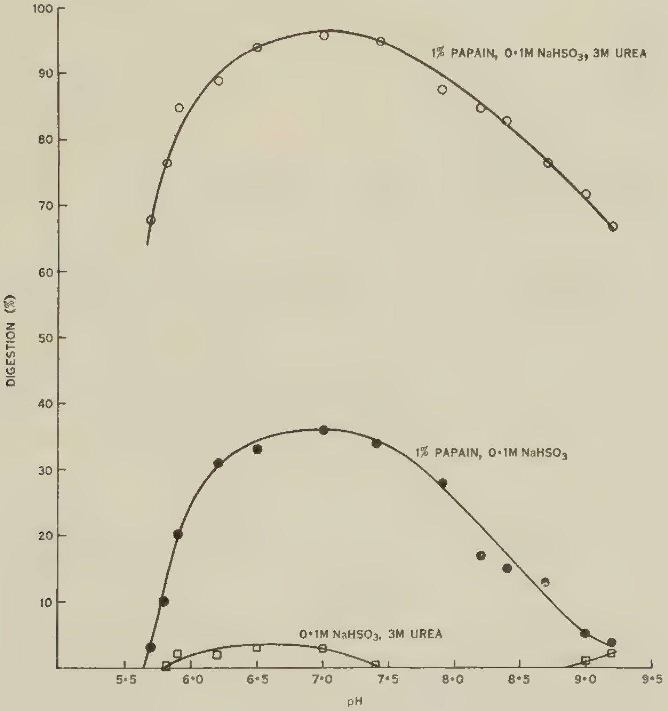

Fig. 2.—pH-wool keratin digestion in sodium bisulphite solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

Sodium bisulphite and sodium thioglycollate together in the papain-urea solution increased digestion as shown in Figure 6. In this experiment the pH maxima were most clearly apparent after incubation for only 1 hr. at 50°C.

To enable the course of wool digestion in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea to be conveniently followed and compared with that previously reported for papain-bisulphite-urea (Lennox 1952) the initial pH was adjusted to 8.6. The amount of digestion after 6 hr. at 50°C. at this pH value was similar to that observed when bisulphite was used at pH 7.0, but the digestion proceeded more rapidly in the early stages of incubation (Fig. 7).

The change in the rate of digestion in the presence of thioglycollate is probably due to a fall in pH value to 7.0, which occurred within the first 2 hr.

The effect of pH on the formation of sulphydryl groups after incubation of 0.020 g. samples of wool for 1 hr. at 50°C. in 0.6 ml. portions of 0.1M solutions of the compounds previously tested in the papain-urea solutions was measured by the iodosobenzoic acid method and is shown in Figure 8. After incubation the wool was immersed for several hours in two changes each of 20 ml. distilled water (which had been stored under hydrogen) followed by one rinse in ethanol. In this way the reagents were removed with minimum risk of oxidizing the wool. Excess liquid was removed after each immersion by repeatedly squeezing the samples between filter paper. Softening of the wool became evident as the pH value of the thioglycollate solutions increased above 9.0. The weight of wool was reduced in thioglycollate to 0.019 g. at pH 10.0 and to 0.017 g. at pH 10.5; at the latter pH most of the fibrous structure disappeared and the product became plastic. Hardening occurred on immersion in ethanol. In the cysteine solutions slight softening of the fibres was detected at pH 10.0 and 10.5. The form of the pH-thioglycollate reduction curve is similar to that reported by Patterson *et al.* (1941) although, because of the milder conditions employed, the extent of reduction is less. The formation of sulphydryl groups in the presence of potassium cyanide has been confirmed by increasing the concentration of this reagent to 1.0M, adjusting the pH value to

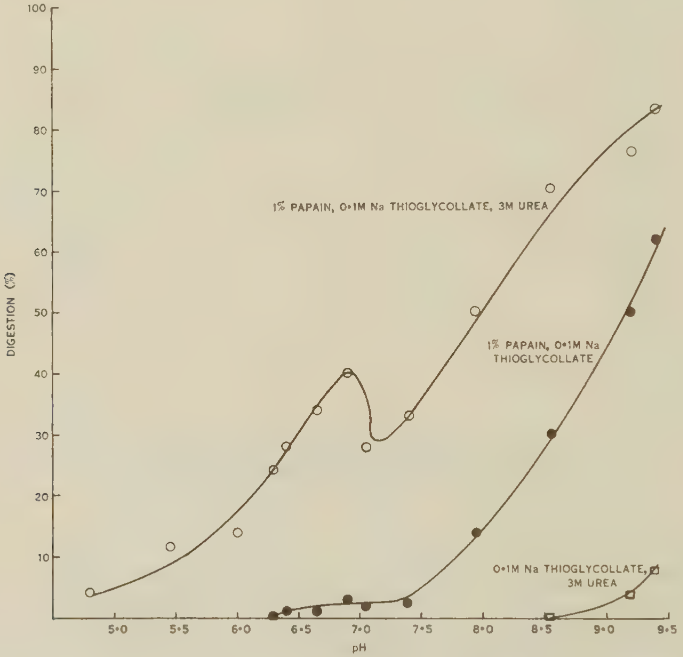

Fig. 3.—pH-wool keratin digestion in sodium thioglycollate solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

10.5, and incubating as before for 1 hr. at 50°C. Under these conditions the concentration of sulphydryl sulphur formed was doubled. Further confirmation was obtained by incubating in potassium cyanide solutions as above, and staining with sulphydryl group reagents such as sodium nitroprusside. Such methods also showed that material containing free sulphydryl groups was extracted from the wool by the potassium cyanide solution.

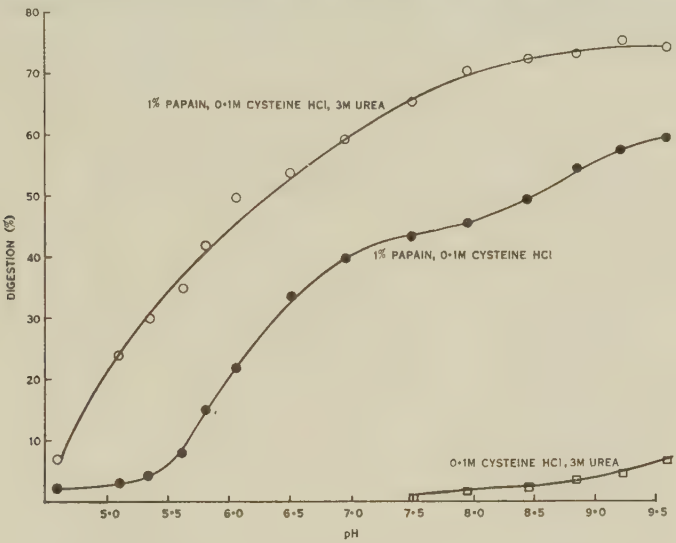

Fig. 4.—pH-wool keratin digestion in cysteine hydrochloride solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

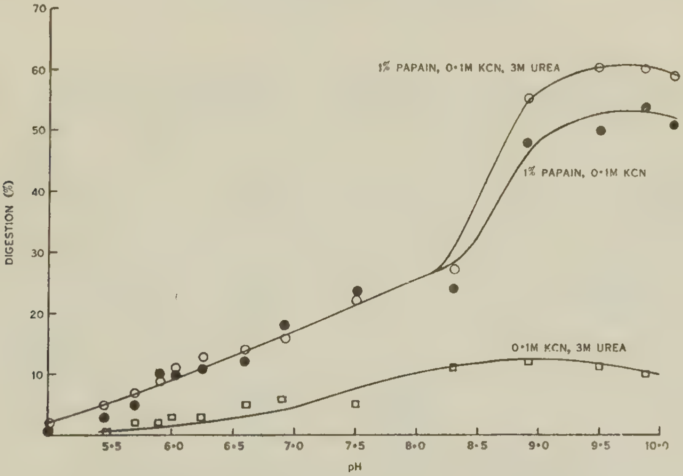

Fig. 5.—pH-wool keratin digestion in potassium cyanide solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

After reduction of wool at various pH values in 0.1M thioglycollate, as in the above experiment, incubation for 1 hr. at 50°C. in 1 per cent. papain, 3M urea at pH 7.0, produced the digestion shown in Figure 9. Prior reduction apparently predisposed the wool to subsequent attack by papain-urea solution, but it will be noted that the digestion was less than that produced by the simultaneous action of papain, thioglycollate, and urea. Transfer from the 0.1M thioglycollate solution to the papain-urea probably allowed reoxidation of some

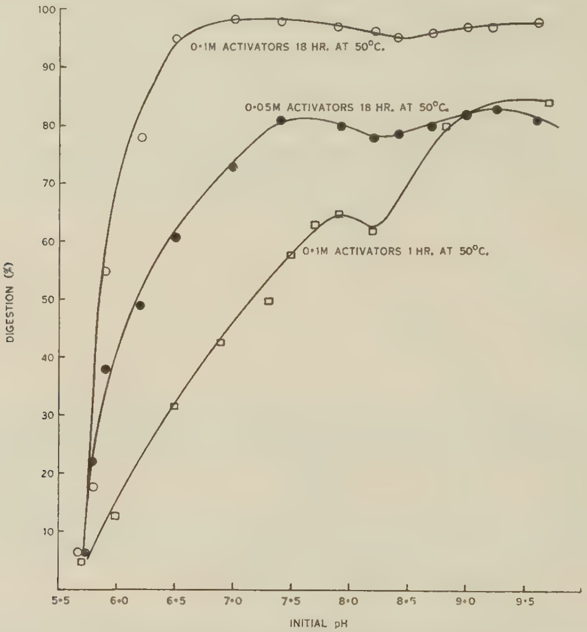

Fig. 6.—pH-wool keratin digestion in papain-urea solutions containing also the mixed activators sodium bisulphite and sodium thioglycollate.

Table 1

WOOL DIGESTION AND SULPHYDRYL SULPHUR IN WOOL AFTER INCUBATION FOR 1.5 HR. AT 50°C. IN THIOGLYCOLLATE SOLUTIONS WITH AND WITHOUT PAPAIN AND UREA

Wool Incubated in Solution Containing			Incubated at pH 7·0		Incubated at pH 8·0	
1 Per cent. Papain	0·1M Sodium Thioglycollate	3M Urea	Digestion (%)	Sulphydryl S (%)	Digestion (%)	Sulphydryl S (%)
—	+	—	Nil	0·83	6	1·38
—	+	+	Nil	1·03	4	1·47
+	+	—	21	1·16	66	1·66
+	+	+	50	1·41	82	1·06

of the sulphydryl groups in wool to disulphide bonds. The enzyme and urea would then be unable to exert as great a disruptive action on the keratin structure as when they act in the presence of 0.1M thioglycollate. Similar treatment in sodium bisulphite solutions failed to predispose the wool to subsequent digestion in papain-urea.

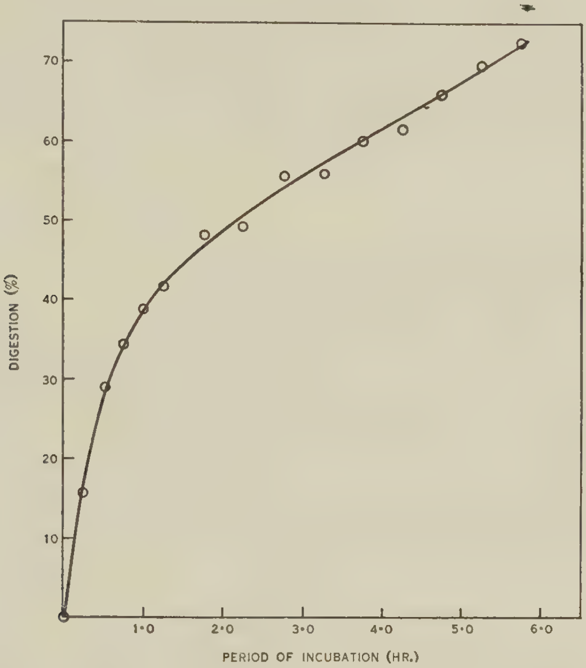

Fig. 7.—Progress of wool keratin digestion in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea during incubation at pH 7.5 (initial pH 8.6).

Greater reduction of disulphide bonds by thioglycollate in the presence of urea or papain than in their absence is apparent from the results in Table 1. The relatively low concentration of sulphydryl groups in the small residue remaining after digestion in papain-thioglycollate-urea at pH 8.0 indicates that keratin passing into solution was probably rich in these groups.

The pH-digestion curve for sheepskin collagen in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea resembles that observed for wool and shows a pH optimum at approximately 7.0 (Fig. 10). The curve representing the digestion of collagen in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea differs markedly from the corresponding curve for wool keratin. Digestion was maximal in acid solutions and it diminished as the pH exceeded 7.5.

The initial pH values of the enzyme solutions in both series of tests were adjusted to values separated by 0.5 pH units between 4.0 and 10.5. The pH values reported in Figure 10 for collagen digestion, which were measured after the completion of incubation, reveal marked changes in some solutions and entirely different changes for the two activators. The existence of four pH maxima in the thioglycollate series was confirmed in a second experiment.

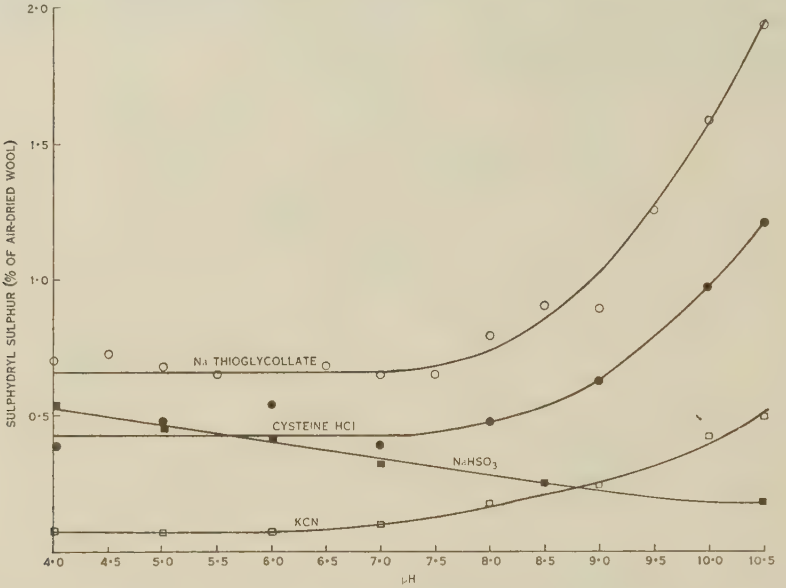

Fig. 8.—Effect of pH on reduction of disulphide bonds in wool by 0.1M solutions of compounds that rupture disulphide bonds. Incubated for 1 hr. at 50°C.

IV. Discussion

Schöberl and Hamm (1948) observed maximum digestion of wool in papain-cyanide solution at pH 9. Up to 40 per cent. was digested in 18 hr. at 60°C. and by repeated use of fresh papain solution the digestion was increased to 80 per cent. In the present study the pH optimum was shown to be 9.5-10.0.

Cuthbertson and Phillips (1945) showed that incubation of wool for 16.5 hr. in 1 per cent. potassium cyanide at 66°C. converted nearly all the combined cystine into combined lanthionine, the sulphur released during the process forming potassium thiocyanate. The development of a reddish brown colour during the digestion of wool at pH values above 8.5 in papain-cyanide, in the presence of urea, though to a lesser extent in its absence, may be due to the formation of a compound containing thiocyanate. Unlike ferric thiocyanate, however, it was not soluble in ether, even after precipitation of protein from the digest with trichloracetic acid. The pigment passed slowly through cellophane during dialysis and when subjected to paper electrophoresis it moved towards the anode more rapidly than the high molecular weight components in the digest, which stain with bromphenol blue.

It will be noted that the relative effectiveness of the various compounds in producing sulphydryl groups in wool resembles their contribution to wool digestion when incorporated in papain-urea solutions (Fig. 1) and incubated under identical conditions. In comparing the disulphide bond-rupturing agents allowance must be made for the liberation by sodium bisulphite of only one sulphydryl group for each disulphide bond split, the other half of the cystine residue being converted to S-cysteine sulphonate (Elsworth and Phillips 1938). The same is probably true of the action of potassium cyanide. According to Cuthbertson and Phillips (1945), during the production of lanthionine cross-linkages in the presence of cyanide, one-half of each cystine residue yields a cysteine residue, which is presumably the source of the sulphydryl groups reported in Figure 8. In contrast to bisulphite and cyanide, thioglycollate and cysteine produce two sulphydryl groups from each cystine disulphide linkage, and if allowance is made for these differences in comparing the results in Figure 8, bisulphite is found to split more disulphide bonds than the other three compounds in acid solution. Above pH 9 however, the relative order is: thioglycollate, cysteine, cyanide, and bisulphite, corresponding to their effects in increasing the papain-urea digestion of wool. Wool is comparatively resistant to papain-cyanide-urea solutions, even at alkaline pH values (Fig. 1), and this may be due to the formation of lanthionine cross-linkages. Comparison of the results in Figures 1 and 8 also suggests that the rupture of disulphide bonds should exceed a threshold value before wool digestion becomes appreciable. For thioglycollate and cysteine this corresponds to approximately 0.7 per cent. sulphydryl sulphur.

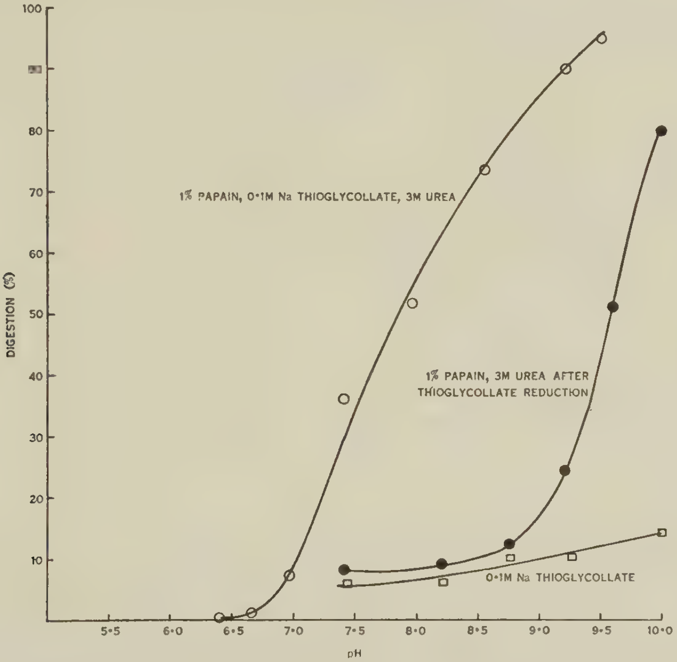

Fig. 9.—Effect of prior reduction of wool in thioglycollate at pH values shown on subsequent digestion by papain-urea at pH 7.0. Incubated for 1 hr. at 50°C.

Further evidence that the action of the disulphide bond-rupturing agents on wool in the presence of papain and urea is not confined to the activation of papain, but also affects keratin directly, was shown by substituting a sample of fully active papain for the material normally used. When tested in solution containing 3M urea but no disulphide bond-rupturing agent, both samples of papain produced approximately 6 per cent. digestion during 6 hr. at 50°C., but when sodium bisulphite was added to a final concentration of 0.1M, digestion exceeded 70 per cent. Failure of ascorbic acid to increase the digestion of wool by papain-urea may be related to its comparatively high E'_0 at pH 7 which has been reported to be + 0.060 V. (Hellerman 1939); the corresponding values for cysteine and thioglycollic acid are approximately − 0.3 V. (Ryklan and Schmidt 1944).

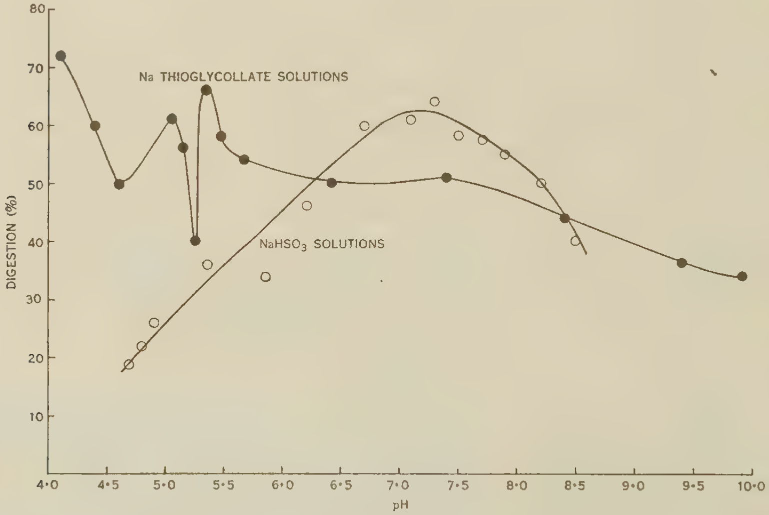

Fig. 10.—Digestion of skin collagen in papain-urea solutions activated with sodium bisulphite or sodium thioglycollate. ○ Incubated for 1.25 hr. at 35°C. in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea. ● Incubated for 0.75 hr. at 35°C. in 1 per cent. papain, 0.1M Na thioglycollate, 3M urea.

Since collagen contains no cystine the main role of sodium bisulphite and sodium thioglycollate during digestion of this protein in solutions containing also papain and urea would be that of papain activation.

V. Acknowledgment

The authors are indebted to Mr. T. A. Pressley for preparing the skin collagen used in this investigation.

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A CRITICAL STUDY OF THE USE OF PAPER ELECTROPHORESIS FOR SEPARATING PROTEINS AND MEASURING THEIR ISOELECTRIC POINTS

By E. F. Woods* and J. M. Gillespie*

[*Manuscript received September* 23, 1952]

Summary

A study has been made of the factors affecting the migration of proteins during electrophoresis on paper strips held between glass plates. A major difficulty in interpreting results is caused by movement of liquid in the paper from anode to cathode, the rate of flow diminishing from the anode end to the cathode end. Even with non-uniform liquid flow of this type it is possible to choose a path such that migration of an ion is linear with respect to both time and potential gradient.

Proteins having high isoelectric points are strongly absorbed on the paper at all pH values, and other proteins are absorbed at pH values below their isoelectric points.

After applying corrections for liquid flow, the mobility of bovine plasma albumin on paper has been shown to approximate that observed in free electrophoresis. The isoelectric point in buffers of ionic strength 0.025 was found to be at pH 4.65. The isoelectric points of the proteases and esterases produced by the mould *Aspergillus oryzae,* as measured by paper electrophoresis are: esterases 3.9, 5.1; proteases 4.2, 5.1. The pH-mobility curve of one of the mould proteases is identical with that of one of the esterases.

I. Introduction

The technique of electrophoresis on filter paper has found great success recently in the examination of mixtures of inorganic salts (McDonald, Urbin, and Williamson 1950), amino acids (Wieland and Fischer 1948; McDonald, Urbin, and Williamson 1951*a*, 1951*b*), of normal and pathological sera (Durrum 1950; Turba and Enenkal 1950; Grassman and Hannig 1950), and of mixtures of enzymes (Mills and Smith 1951; Gillespie, Jermyn, and Woods 1952).

Most workers have developed their own design of equipment for filter paper electrophoresis and amongst the most satisfactory are those due to Cremer and Tiselius (1950) and Kunkel and Tiselius (1951). These workers have also been responsible for developments concerning the theoretical basis of the subject.

In this laboratory the technique has been successfully used in studies of enzymes in culture filtrate from a strain of *Aspergillus oryzae* (Gillespie, Jermyn, and Woods 1952). Most of this work was concerned with the elucidation of the composition of the mixture of enzymes with a view to achieving complete separation of each component so that its concentration and specific activity could be measured. In the present study a critical examination has been made

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of the factors affecting the migration of proteins on paper, in order that measurements could be made of the mobilities and isoelectric points of the various components, which is normally accomplished with larger samples of protein solution by free electrophoresis in the Tiselius apparatus. As reported by earlier workers, one of the major difficulties in paper electrophoresis arises from the fact that a protein spot moving under the influences of an electrical potential gradient on paper is also affected by a complex flow of liquid; the buffer solution moves from anode to cathode, the rate of flow being greatest at the anode end and decreasing towards the cathode. At least two movements of liquid can be recognized, firstly that due to electro-endosmosis producing a flow towards the cathode, and secondly a movement of liquid from each end towards the centre of the paper to replace that lost by evaporation. In order, therefore, to make quantitative measurements of protein migration in an electric field, it is necessary to determine the extent of these liquid movements and apply corrections for them. The first part of this paper is concerned with the measurement of these interfering factors and the second part with the estimation of mobility and isoelectric points of some proteins.

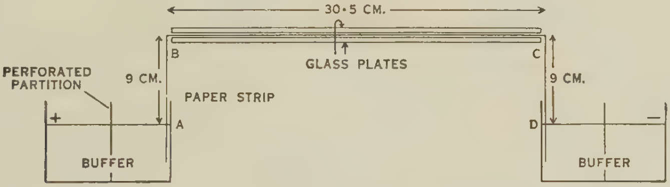

Fig. 1.—Diagram of electrophoresis apparatus.

II. Methods

The apparatus used (Fig. 1) is a modification of that described by Cremer and Tiselius (1950). The filter paper strip* 3.5 cm. wide was placed between two plate glass strips 4 cm. wide and tightly clamped by means of four strong spring clamps. The ends of the strips were immersed in buffer in 1 l. capacity "Perspex" electrode vessels having a perforated partition in the centre of each to keep the ends of the paper strips away from the carbon electrodes. The recommendation of Cremer and Tiselius (1950) to use a non-conducting cooling fluid, such as chlorobenzene, to prevent evaporation was not followed because of the unknown effect of organic liquids on the particular enzymes investigated. It was found that if the exposed length of the paper strip (9 cm.) between the ends of the glass plates and the surface of the liquid in the electrode vessels were shortened the entire strip became very wet during an experiment and inferior separations were obtained.

A well-regulated voltage was applied between the electrodes and the magnitude of this and the total current were constantly monitored. The voltage between *B* and *C* (Fig. 1) was also measured at intervals during each experiment with a voltmeter of 20,000 ohms per volt resistance,† the shunting effect of

* Whatman 3MM filter paper.

† Avo model 8 on the 1000 V. range.

which could be neglected. The voltage between *B* and *C* rose to a maximum and then fell slowly, as shown in Figure 2. This fall in voltage persisted even if the power was switched off overnight and reapplied next day. Similar results were obtained with any voltage within the range 100-1050 V. The total current increased by about 50 per cent. during an experiment and both this and the corresponding voltage drop can be ascribed to an increase in conductivity of the buffer in the filter paper due to evaporation of water. This change of conductivity could be decreased by working at low temperatures or by immersing the filter paper assembly in a non-conducting fluid as recommended by Cremer and Tiselius (1950).

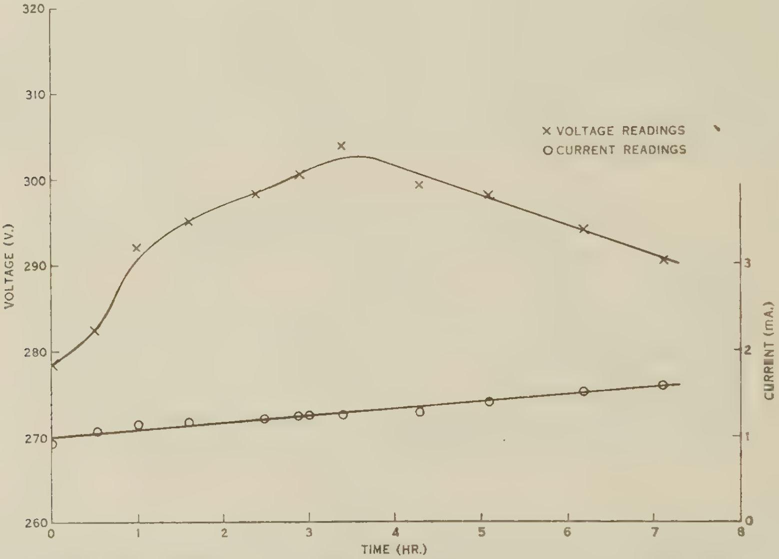

Fig. 2.—Variations in voltage between *B* and *C* (Fig. 1) and total current for a voltage of 400 V. between the electrodes. Veronal buffer Γ/2 0.025 pH 8.6.

The mean value of the voltage measured between *B* and *C* was used in subsequent calculations of potential gradient.

To set up the apparatus, the paper strips were wetted with the buffer solution and blotted to remove excess liquid, and 0.015 ml. of a solution containing between 2 and 10 per cent. protein was applied to a marked position on each strip. They were clamped between the glass plates, the ends were immersed in buffer in the reservoirs, and the desired potential was then applied. After the required period the papers were removed from the apparatus, stained with bromphenol blue (Durrum 1950), and the distance of migration from the point of application of the protein to the most intensely stained portion of the spot was measured. If the protein had enzymic properties, the filter paper strips were cut transversely at 1-cm. intervals along the length and extracts of these portions were tested for specific enzyme activities.

As a convenient standard protein, crystalline bovine plasma albumin (Armour) was used to determine the influence of time and potential gradient on distance moved, whilst an electrodialysed culture filtrate from *A. oryzae* (Gillespie and Woods, unpublished data) was used for experiments with enzymes. Protease activities were determined using sulphanilamide-azocasein as substrate at pH 8 (Charney and Tomarelli 1947). The method used for the determination of esterase was based on that of Huggins and Lapides (1947) using *p*-nitrophenyl acetate as substrate at pH 7 and 37°C.

The ionic strength of the buffers employed (0.025) was sufficiently low to minimize heating by the current yet high enough to ensure adequate buffering capacity. Except where otherwise indicated, measurements were made at 20-25°C.

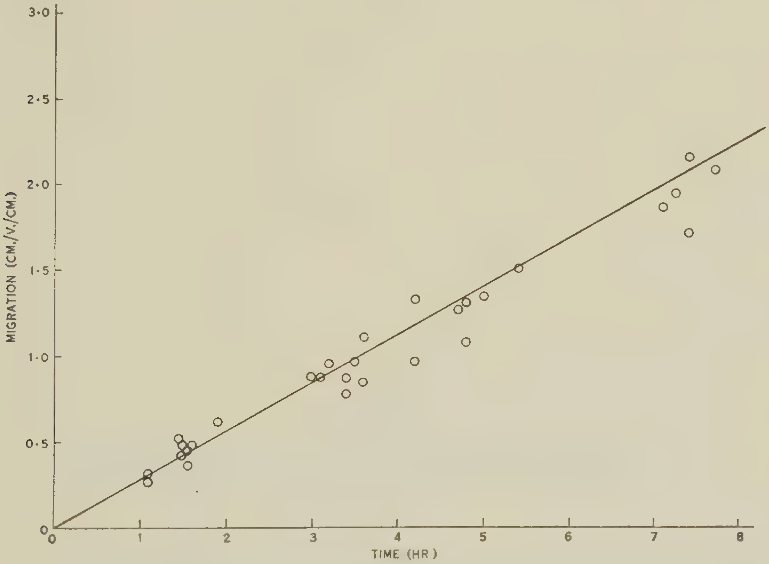

Fig. 3.—Movement of bovine plasma albumin as a function of time. Veronal buffer Γ/2 0.025 pH 8.6.

III. Examination of Factors Affecting Movement on Paper

(*a*) *Effect of Time and Electrical Potential Gradient on Migration*

Figure 3 shows that only an approximate straight-line relation exists between migration and time with bovine serum albumin in veronal buffer at pH 8.6. A similar deviation was found for the relation between rate of movement and potential gradient. These deviations were found to vary with the point of application of the protein on the paper, and the path over which it moved. The protein moved faster on the anode side of the centre of the paper strip than on the cathode side. Durrum (1950, 1951*a*, 1951*b*) has discussed these deviations from linearity and has reported the phenomenon of "mobility equilibrium," claiming that an ion moves to an equilibrium position on the paper irrespective of its point of application owing to variation in the liquid flow along the paper. Experimentally it was found possible to overcome these deviations. If a protein spot was placed at 6 cm. from the cathode end of the glass strip and allowed to migrate 18 cm. until it was 6 cm. from the anode end, then a straight-line relation was observed between distance moved and potential gradient. This can be seen in Figures 4 and 5. It seems that when a protein moves the same distance on either side of the centre of the strip, variations in liquid flow due to evaporation can be neglected. However, the mobilities must be corrected for liquid movement due to electro-endosmosis.

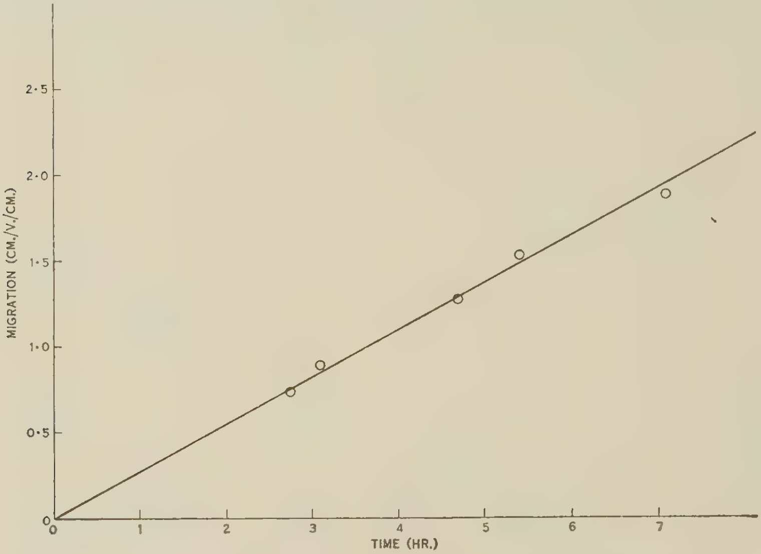

Fig. 4.—Movement of bovine plasma albumin over a fixed 18 cm. path as a function of time. Veronal buffer Γ/2 0.025 pH 8.6.

(*b*) *Investigation of Liquid Flow along the Paper*

Liquid flow was investigated by placing spots of glucose at intervals along the paper, as recommended by Kraus and Smith (1950) and Schwarz (1951), and determining their positions at the end of an experiment by heating the paper at 110°C. to char the glucose and produce brown spots. The distance moved towards the cathode from the point of application was plotted against the initial position of the spot on the paper. Displacement of glucose provides an accurate measure of the movement of liquid in the paper.

Figure 6 shows the variation in rate of liquid flow along the paper for veronal buffer of 0.025 ionic strength in some typical experiments. It can be seen that the rate of liquid flow progressively increased from the cathode end of the paper to the anode end approximately in proportion to the distance from the cathode. The results obtained for the migration of bovine plasma albumin, reported in this paper, support the view that the liquid flow is greater on the anode side of the centre than on the cathode side. These results can be explained on the basis of two combined liquid movements, that due to electro-endosmosis, which causes a flow of liquid from anode to cathode, and that caused by evaporation resulting in a movement of liquid from each end to the centre. It has been observed that considerable shifts of liquid into the paper occur without the passage of the electric current. These movements, which continue for many hours, are symmetrical; they decrease from each end towards the centre and depend to some extent on the method of wetting the paper. It has been found that the liquid flow gradient is a function of time, potential gradient, position of the spot on the paper, method of wetting the paper, the extent of evaporation (which depends on the particular experimental technique adopted), and the pH and ionic strength of the buffers used.

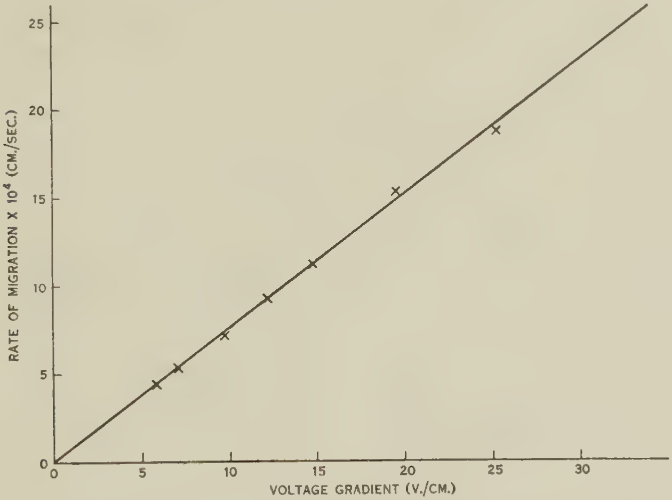

Fig. 5.—Movement of bovine plasma albumin over a fixed 18 cm. path as a function of potential gradient. Veronal buffer $\Gamma/2$ 0.025 pH 8.6.

The effect of potential gradient on liquid flow is shown in Figure 6. It will be seen that there is a marked increase in liquid flow on increasing the voltage from 9.5 to 19.5 V./cm. At 19.5 V./cm. a spot of glucose placed in the centre of the paper moves 8.2 cm. towards the cathode in 5 hr. This corresponds to a mobility of 2.3×10^{-5} cm.^{2}/V./sec., and so a negatively charged substance with this mobility would remain stationary on the paper. The migration velocity of an ion at any point on the paper will be the resultant of the liquid movement at that point and the electrophoretic mobility. Experiments have shown that the liquid flow at a particular position changes slightly with time, and consequently only under certain operating conditions would it be possible for an ion to reach a stationary position on the paper, irrespective of the starting point. These conditions apparently apply in Durrum's experiments (1951*b*). In the experiments demonstrating the attainment of "mobility equilibrium" Durrum used a potential gradient of 35 V./cm. and he supported his strips over a glass rod in a closed vapour space. Moreover the total distance between the electrodes was 30 cm. as compared to more than 50 cm. in the present investigation. We have found that if the distance *BC* (Fig. 1) is too short liquid flow into the paper is excessive. Durrum (1951*b*) has also reported that increasing the apical angle decreased the flow gradient and gave better resolution in this method, and hence the technique used in the present study should greatly reduce liquid flow. The contradiction between the finding of McDonald, Urbin, and Williamson (1951*a*, 1951*b*) and of McDonald *et al.* (1951) and that of Durrum (1950, 1951*a*, 1951*b*) is also probably explained by differences in the techniques used. The potential gradient, distances between electrodes, volume of enclosed vapour space, and the method of supporting the strip will all influence the volume of liquid flowing into the paper from the electrode vessels. In the present investigations it has been possible to obtain a linear migration of protein with time and potential gradient, as shown also by McDonald *et al.* (1951), simply by choosing a suitable migration path even though the liquid flow was not uniform along the paper. In determining the isoelectric points of amino acids, McDonald *et al.* (1951) made no correction for liquid flow although this must have occurred in their experiments. Electro-endosmosis always occurs in such a system and corrections must be applied to compensate for this even if there are no complications due to evaporation. In electro-endosmosis only, the liquid flow should be uniform along the paper and, in the absence of adsorption, movement of ions will be directly proportional to time and potential gradient.

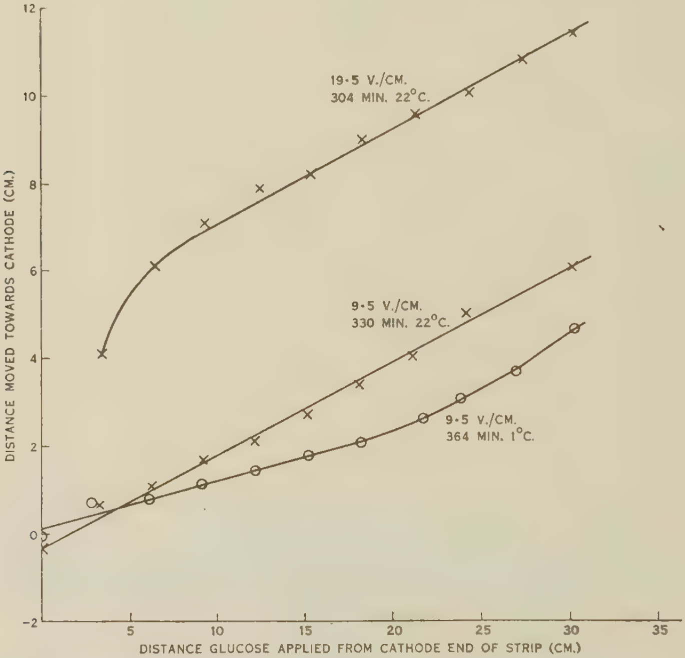

Fig. 6.—Liquid flow in paper electrophoresis as indicated by movement of glucose at various points along the paper. Veronal buffer Γ/2 0.025 pH 8.6.

In conformity with the experience of other workers, it has been observed in this laboratory that evaporation is diminished by working at a lower temperature. This is evident from Figure 6 where the liquid flow is shown for an experiment carried out at 1°C., but even at this temperature evaporation was not completely eliminated and non-uniformity of liquid movement was still observed.

(*c*) *Adsorption of Proteins on Paper*

For satisfactory separations of protein mixtures on paper and for the determination of ionic mobilities, adsorption on the paper should not occur. Brattsten and Nilsson (1951) found that the red-coloured algal protein, phycoerythrin, was strongly adsorbed on filter paper. Schwarz (1951) found that the pH-mobility curve of serum proteins was flatter below pH 5.0 than the corresponding curve obtained in free electrophoresis, and he attributed this to adsorption of the positively charged protein on the negatively charged filter paper. In this laboratory adsorption on paper has been found to occur very strongly during the paper electrophoresis of proteins on the acid side of their isoelectric points. Proteins and polypeptides with high isoelectric points were also strongly adsorbed at pH values between 2 and 11. Figure 7 shows the type of pattern obtained with papain in buffer at pH 4.8. This enzyme has an isoelectric point of about pH 9.0 (Balls and Lineweaver 1939). The basic polypeptide secretin,* and the basic protein, salmine, behaved similarly to papain on paper electrophoresis. Even at pH values above the isoelectric points, adsorption still occurred. These proteins contain a high proportion of arginine residues which would be ionized to give a net positive charge up to pH 12.5, which is the pK value for the guanidine residue (Cohn and Edsall 1943). Paper electrophoresis of basic proteins at pH values sufficiently high to remove the charge on the side chains of arginine presented technical difficulties for the filter paper became weak and difficult to handle and the high conductivity of the buffers resulted in excessive heating.

In the paper chromatography of amino acids, similar streaking has been found to be due to the presence of heavy metals (Consden, Gordon, and Martin 1944), and this was eliminated by washing the paper with dilute acid or more effectively by treating it with a metal-chelating agent, such as 8-hydroxyquinoline or ethylene diamine tetra-acetate. Attempts to eliminate adsorption during paper electrophoresis of papain by either of these means were unsuccessful. Bovine plasma albumin showed strong adsorption on filter paper below pH 4.5 and in the separation of the enzymes of *A. oryzae* by paper electrophoresis at several pH values, adsorption by the paper of some of the components of the mixture became pronounced at pH values below 5.0. Figure 8 shows the patterns obtained by staining with bromphenol blue after electrophoresis of the electrodialysed enzyme preparation at several pH values. Hall and Wewalka (1951) consider that the protein spots which Franklin and Quastel (1949, 1950) obtained by chromatography of plasma were not different chemical entities but artifacts, the nature of which depended on the properties of the proteins in contact with the paper. The same considerations may also apply to the movement of proteins on paper under the influence of an electric field. Hence in any separation obtained by paper electrophoresis it is desirable to eliminate the possibility of adsorption and test the separate identity of the components by some other means. It has already been shown (Gillespie, Jermyn, and Woods 1952) that many of the enzymes in the culture filtrate from *A. oryzae* are multiple in nature. Later work has shown that the various components separated by paper electrophoresis are not artifacts, for they retain their separate identities on fractionation with ethanol under conditions of low ionic strength and low temperature, and furthermore two of the protease components have differing specificities (Gillespie and Woods, unpublished data) as do many of the β-glucosidases (Jermyn 1952).

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* Sample kindly prepared by Mr. G. E. Rogers by a modification of the method of Greengard and Ivy (1938).

IV. APPLICATION OF THE METHOD TO THE DETERMINATION OF THE MOBILITIES AND ISOELECTRIC POINTS OF BOVINE PLASMA ALBUMIN AND SOME MOULD ENZYMES

From the results given in Figures 4 and 5 a mean apparent mobility of 7.6×10^{-5} cm.^{2}/V./sec. is derived for bovine plasma albumin in 0.025 ionic strength veronal buffer at pH 8.6. This value was corrected for liquid flow due to electro-endosmosis and evaporation by running at the same time as the protein, parallel strips with spots of glucose at 3 cm. intervals along the paper, and taking the mean value of the liquid flow at the beginning and end of the path of migration. This only gives an approximation for the liquid flow; it is not practicable to apply an accurate correction as the protein moves along a path with a varying rate of flow of liquid. In systems where evaporation occurs, the ionic composition will also vary along the paper (Durrum 1951*b*) and this will cause variations in the calculated mobilities for which it is not possible to apply any correction. Four replicate determinations of the corrected mobility of bovine plasma albumin gave a mean value of 9.1×10^{-5} cm.^{2}/V./sec. Thus liquid movement accounts for nearly 20 per cent. of the migration velocity in this case. By electrophoresis in the moving boundary apparatus at 0°C. in veronal buffer of 0.1 ionic strength a value of 6.5×10^{-5} cm.^{2}/V./sec. was obtained by Alberty (1948) for the mobility of the same protein. Since the migration velocity increased by about 2 per cent. per 1°C., the value for bovine plasma albumin at 23°C. is approximately 9.5×10^{-5} cm.^{2}/V./sec. The value obtained in paper is thus in close agreement with that obtained in free electrophoresis.

Both the work of Kunkel and Tiselius (1951) and that described here, although using slightly different assumptions as to the factors operative in the process, have given approximately correct values for the mobilities of plasma albumins. On the one hand Kunkel and Tiselius introduce the factor l/l' which is claimed to take into account the tortuous path a protein may follow in its passage through the paper. On the other hand we have made a correction for the velocity gradient along the paper which arises from non-uniform liquid flow. This does not appear to have been taken into account by Kunkel and Tiselius for the only liquid flow movement considered by them was that de-

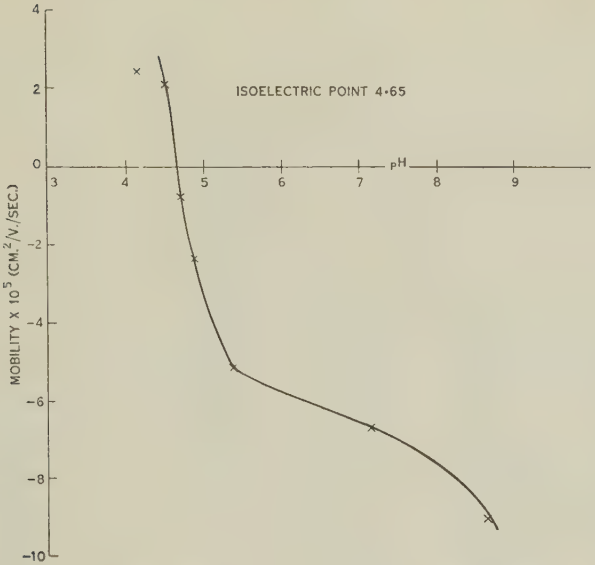

Fig. 7.—pH-mobility curve for bovine plasma albumin.

fined by the shift of a single dextran spot from the origin. The fact that the two methods give correct mobilities suggests that all the factors operative in filter paper electrophoresis are not as yet fully defined and understood.

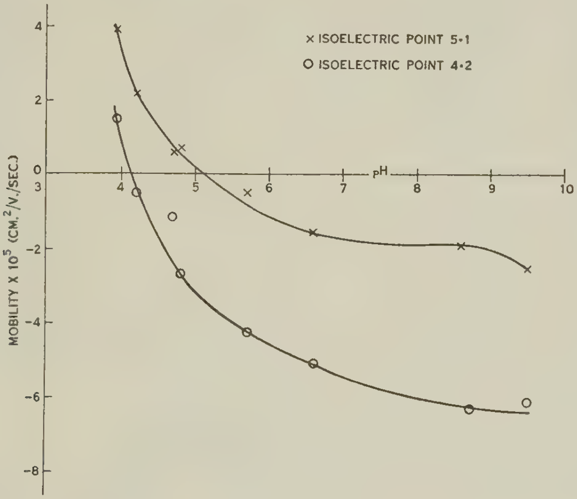

Fig. 8.—pH-mobility curves for two proteases from *A. oryzae*.

Having achieved a method for measuring mobilities it is possible to extend the work to the calculation of isoelectric points of proteins both in the pure state and in admixture with other proteins. The mobility of bovine plasma albumin was measured at a number of pH values and the results are plotted in Figure 7. It can be seen that the isoelectric point obtained, namely pH

4.65, is in good agreement with the most recently determined value (4.7). This procedure was then used for determining the isoelectric points of some of the proteases and esterases of the mould enzyme mixture. Paper electrophoresis experiments were run on electro-dialysed mould enzyme at several pH values and the enzyme activities of aqueous extracts of strips of the paper were determined. The pH-mobility curves are shown in Figures 8 and 9. The esterases have isoelectric points of 3.9 and 5.1 and the proteases 4.2 and 5.1. One of the proteases and one of the esterases move together at all pH values and have identical pH-mobility curves within the limits of the accuracy of the method. It was not possible to employ lower pH values because of the complications due to adsorption, as evidenced by the patterns in Plate 1, Figure 2, and also because at pH values below 4 the protease activity is lost rapidly (Crewther and Lennox 1953). The values obtained for the isoelectric points are in agreement with the observation that crystals, containing protease (Crewther and Lennox 1950, 1953) from *A. oryzae* culture filtrate, form in ammonium sulphate solutions over the pH range 4.0-5.2 but most readily at pH 4.6. Paper electrophoresis showed that these crystals contained the protease and esterase of isoelectric point 5.1 but only traces of the protease of isoelectric point 4.2.

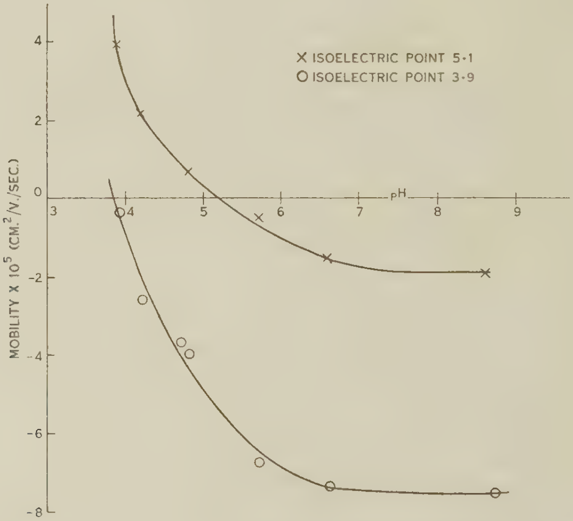

Fig. 9.—pH-mobility curves for two esterases from *A. oryzae*.

V. Acknowledgments

The authors wish to record their thanks to Mrs. H. M. Forss for carrying out the esterase determinations, and to Mr. K. I. Wood for technical assistance.

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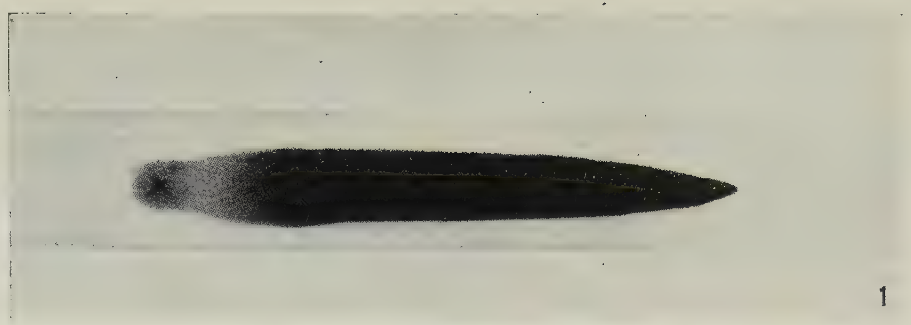
PAPER ELECTROPHORESIS OF PROTEINS



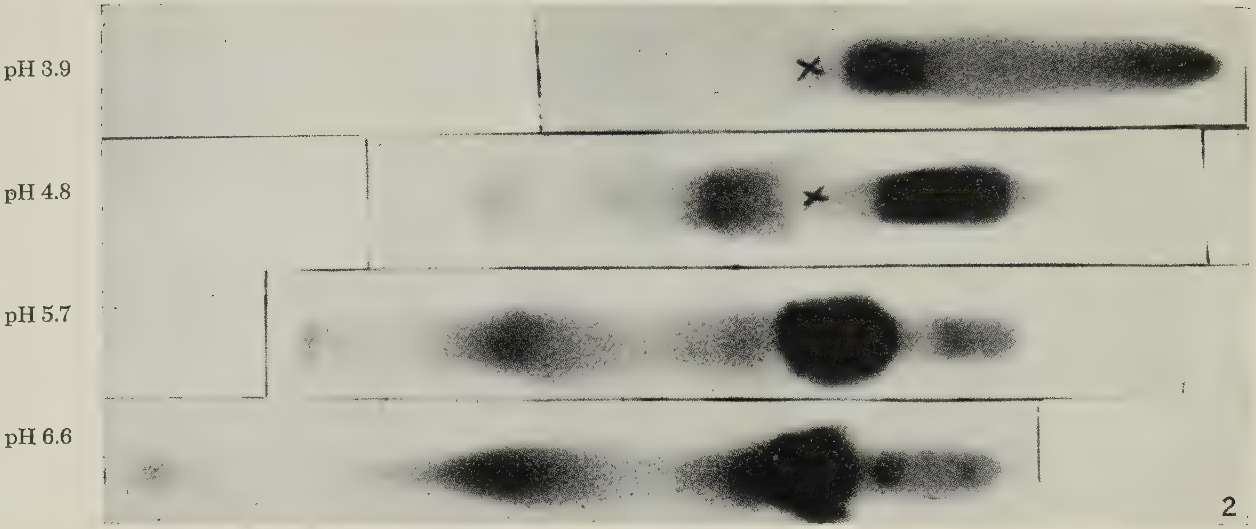


Fig. 1.—Paper electrophoresis of water-soluble fraction of papain in acetate buffer Γ/2 0.025 and pH 4.8.
Fig. 2.—Paper electrophoresis of enzymes of *A. oryzae* at various pH values and buffer Γ/2 0.025, 9.5 V./cm. for 6 hr. Cathode at right end of both figures.

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RUMINAL FLORA STUDIES IN THE SHEEP

IV. THE INFLUENCE OF VARYING DIETARY LEVELS OF PROTEIN AND STARCH UPON DIGESTIBILITY, NITROGEN RETENTION, AND THE FREE MICROORGANISMS OF THE RUMEN

By V. J. Williams,* M. C. Nottle,* R. J. Moir,* and E. J. Underwood*

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Summary

Data are presented from a replicated feeding trial with sheep, designed to determine the influence of varying dietary intakes of starch and protein upon digestibility, protein utilization, and the numbers and types of "free" bacteria in the rumen.

The main trial contained nine diets composed of oaten chaff to which wheaten starch and wheat gluten were added so as to provide three levels of starch (nil, medium, and high) and three levels of protein (low, medium, and high).

The concentration of free microorganisms in the rumen was found to be markedly affected both by the levels of dietary protein and by the levels of dietary starch, but the extent to which either of these constituents affected this concentration was greatly influenced by the proportion of the other constituent present in the diet.

In low-protein diets, increases in the intake of starch were found to result in a highly significant reduction in the rumen counts as well as a marked change in the types of organisms present. Where the protein contents of the diets were higher no such effects were evident.

In diets containing medium or high levels of starch, increases in the intake of protein were found to result in highly significant increases in the rumen counts. No such effects were evident in starch-free diets.

At all levels of starch the addition of protein was found to increase significantly the digestibility of the dry matter of the diets and the true digestibility of the protein as well as the rumen counts, but the addition of starch to low-protein diets markedly reduced these counts without influencing significantly the digestibility of the dry matter.

These findings are compared with the results of Moir and Williams (1950) and others, and the conclusion reached that the proportion of *total*, as distinct from *added*, dietary protein converted to bacterial protein in the rumen is not relatively constant, as earlier claimed, but diminishes sharply as the intake of protein increases.

I. Introduction

In an earlier experiment by the authors (Moir and Williams 1950) an extremely high correlation ($r = +0.98$) was found between the levels of protein intake and the concentrations of free microorganisms in the rumen. The basal ration in this experiment consisted of oaten hay and wheaten starch and contained only 3.7 per cent. protein (N × 6.25). The protein content of the

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basal ration was increased by substituting increasing proportions of the starch with equal weights of casein until a ration containing 18 per cent. protein was obtained. In this way the protein content of the five rations used varied from 3.7 to 18 per cent. but their dry matter, crude fibre, and gross energy contents remained unchanged. It is clear, however, that the higher the protein content of rations the lower were their starch contents. It may therefore be asked whether the concentrations of ruminal organisms obtained were a reflection of the higher protein levels of the substituted basal rations or whether they were also influenced by the lower starch levels of these rations. In this paper are presented the results of experiments in which both starch and protein were varied independently.

Considerable overseas evidence exists concerning the influence of supplements of protein and of starch and other carbohydrates upon the digestibility of roughages by ruminants but the effects produced have seldom been related to changes in the ruminal population. Gallup and Briggs (1948) increased the dry matter digestion of prairie hay with cottonseed meal, although Watson *et al.* (1947) found no such effect from additions of soybean oil meal to timothy hay. Burroughs *et al.* (1949*a*) showed that various high-protein feeds restored the digestibility of poor-quality roughages, in the presence of a high proportion of starch. Protein supplements without added starch produced no such effects. In a later study these workers (Burroughs *et al.* 1950) confirmed their earlier findings and extended them to include some bacteriological studies. They found a close correlation between the numbers of ruminal bacteria and the degree of roughage digestion. Where the roughage (ground corncobs and chopped alfalfa hay 4:1) was fed alone digestion was relatively high and the ruminal population high. Addition of starch alone resulted in a marked depression of both digestibility and ruminal population. Addition of starch and casein together restored the digestibility of the roughage and the numbers of ruminal bacteria to levels similar to those of the unsupplemented roughage. It is unfortunate that these workers did not include a diet in which the roughage was supplemented with casein only.

Hamilton (1942) showed that the addition of glucose reduced the digestibility of protein and of fibre in rations for sheep. Burroughs *et al.* (1949*b*) obtained a substantial decrease in roughage digestion by steers by the addition of starch to poor-quality roughages but not with good-quality alfalfa hay. They claimed that the higher protein content of the alfalfa was not the factor causing the difference in the starch effects and suggested that "the variable influence of starch upon roughage digestion can best be explained on the basis of adequate or inadequate nutrients (other than protein) for the growth of microorganisms in the alimentary tract." Louw and van der Wath (1943) added meatmeal to a ration of poor prairie hay and obtained a highly significant increase in the numbers of ruminal bacteria. Replacement of varying proportions of meatmeal with maize resulted in minor increases in the counts until the maize supplements reached 150 g. This increased bacterial population did not improve cellulose digestion. Further addition of maize in the absence of meatmeal caused a decline in bacterial numbers and a significant decline in cellulose digestibility notwithstanding the fact that the N intake increased in these latter diets.

II. Experimental

(*a*) *Design of Experiment*

The feeding trial was designed as a balanced lattice, as set out in Table 1, containing four blocks of replicates of each of nine dietary treatments. The treatments were triplicated at random within each block and each triplicate was allotted at random to one of 12 sheep.

Table 1

EXPERIMENTAL DESIGN — ORDER OF FEEDING DIETS

Sheep No.	Diet No.		
339	2	0	1
338	5	3	4
344	6	8	7
337	3	6	0
332	7	4	1
345	5	8	2
340	8	4	0
341	6	5	1
349	7	3	2
342	5	7	0
347	3	8	1
343	4	2	6

(*b*) *Experimental Animals*

Twelve Merino wethers were selected for evenness of age and appearance from a larger group of similar breeding. Each of the experimental animals weighed about 90 lb. and was between 2 and 2½ years of age.

(*c*) *Diets*

The composition of the nine diets used was as set out in Table 2. They were designed to provide three levels of starch and three levels of protein, with oaten chaff as the roughage in all cases.

It was found impossible to restrict the diets to the three main constituents, oaten chaff, wheaten starch, and wheat gluten because of serious difficulties in persuading the sheep to consume the prescribed amounts of certain types of rations and of preventing them from selecting particular parts of others. Accordingly a proportion of wheaten grain (ground) and wheaten bran was included in some of the diets and in some also a small amount of molasses was added as a binding agent. In all diets, except 0, 1, and 2, the concentrates were sprayed with water while being mixed in a concrete mixer and then dried in an oven at 55°C. in a current of air. In diets 1 and 2 the starch was drenched daily as a suspension in its own weight of water because the sheep refused to consume it voluntarily.

It is clear from Table 2 that the diets can be compared in several ways. Thus a comparison of the data from diets 0, 3, and 6 enables an assessment to be made of the influence of three different levels of protein in starch-free or virtually starch-free diets; a comparison of diets 1, 4, and 7 of similar levels of protein at medium starch intakes; and of 2, 5, and 8 of these levels of protein at relatively high starch intakes. Alternatively a comparison of the data from diets 0, 1, and 2 enables an assessment to be made of the influence of three levels of starch in low (3.5-5.2 per cent.) protein diets; of 3, 4, and 5 of similar levels of starch in medium (8.2-10.0 per cent.) protein diets, and of 6, 7, and 8 of these levels of starch in diets of higher (10.6-13.1 per cent.) protein content.

TABLE 2

COMPOSITION AND INTAKE OF DIETS — G. DRY MATTER PER DAY

Diet	Oaten Chaff	Wheat	Wheaten Starch	Wheaten Gluten	Wheaten Bran	Molasses	Ground Limestone	Rock Salt	Dry Matter Intake (g./day)	Percentage N	Percentage Crude Protein	Intake Protein (g./day)	Intake Starch (g./day)
0	270								270	0·83	5·2	14·0	0
1	270		99						369	0·63	3·9	14·2	99
2	270		149						419	0·56	3·5	14·6	149
3	495			41	58		3	3	600	1·60	10·0	60·0	0*
4	495	74	49	41		18	3	3	683	1·48	9·3	63·4	99
5	495	74	99	41		18	3	3	733	1·31	8·2	60·1	149
6	495			80	58		3	3	639	2·10	13·1	83·7	0*
7	495	74	49	80		18	3	3	722	1·83	11·4	82·3	99
8	495	74	99	80		18	3	3	772	1·70	10·6	81·8	149

* Excluding small amount present in wheaten bran.

(d) *Treatment of Animals and Samples*

Each feeding period extended over 24 days, the last 10 days of which constituted the collection period. Between treatments all sheep were given a "stabilizing" period of 24 days on diet 4 (medium protein, medium starch) to ensure a comparable nitrogen status and to minimize any effects of previous treatments on the ruminal population. The sheep were kept in metabolism crates for the treatment periods and in separate pens for the stabilizing periods.

The whole of the prescribed daily ration was fed each day at 9 a.m., except that in rations 1 and 2 the starch was drenched at 10 a.m., i.e. 1 hr. after the chaff was fed. Adequate tap water was before the sheep at all times and the sheep were drenched once a week with a vitamin A and D concentrate. This was carried out at least 7 days before the rumen contents were sampled.

Urine, faeces, and any feed residues were collected daily immediately before feeding and treated as described by Moir and Williams (1950). On the seventh and ninth days of each collection period samples of rumen contents were withdrawn by stomach tube and treated as described by Moir (1951).

The sheep were weighed on the first, thirteenth, and twenty-fourth days of each treatment. The mean of the last two weights was used in the calculation of the biological values of the protein of the diets.

III. Results

The mean results of the digestibility determinations, nitrogen metabolism data, and ruminal microorganism counts are presented in Table 3. The statistical significance of the results of the various treatments was determined and

Table 3

MEAN DIGESTIBILITY, NITROGEN METABOLISM, AND RUMINAL FLORA DATA AT DIFFERENT INTAKES OF PROTEIN AND STARCH

(Each value the mean of 4 sheep)

	Low Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per cu. mm.)
No starch	Diet 0					
	2·2	83	61	96	52	49
Medium starch	Diet 1					
	2·3	85	92	68	58	27
High starch	Diet 2					
	2·2	81	92	78	55	21

	Medium Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per (cu. mm.)
No starch	Diet 3					
	9·6	92	72	42	63	57
Medium starch	Diet 4					
	9·7	97	75	38	67	47
High starch	Diet 5					
	9·5	93	84	33	67	44

TABLE 3 (*Continued*)

	High Protein					
	N Intake (g./day)	True Digest. N (%)	Calc. Biol. Value N	Urinary N/ Absorbed N (%)	Digest. Dry Matter (%)	Rumen Bacteria (millions per cu. mm.)
No starch				Diet 6		
	13·5	96	65	45	69	59
Medium starch				Diet 7		
	13·3	97	70	37	73	58
High starch				Diet 8		
	13·0	94	64	46	72	54

* The individual data from which these means were calculated may be obtained on request from the Director, Institute of Agriculture, University of Western Australia, Nedlands, W.A.

is discussed below. For the concentration of ruminal microorganisms and the true digestibility of the nitrogen of the diets the results were analysed as designed, i.e. as a balanced lattice, but the lattice was not effective for the calculated biological values of the dietary nitrogen and the dry matter digestibilities. These were analysed as a randomized block. In these determinations, the variability between sheep was as small as, or smaller than, the error term. Tests for significance were either by the variance ratio or Student's *t* test.

(*a*) *Concentration of Free Ruminal Microorganisms*

Effect of starch.—In the low-protein diets there is a marked effect of starch. The mean concentration of 49,000,000/cu. mm. in diet 0 is significantly higher than that of diet 1 (27,000,000/cu. mm.) at the 5 per cent. level and of diet 2 (21,000,000/cu. mm.) at the 1 per cent. level. In the medium-protein diets there is apparently a similar, though much less marked effect since the "mean counts" fall from 57,000,000 to 47,000,000 and then to 44,000,000/cu. mm. in diets 3, 4, and 5 respectively. These differences, however, are not significant. In the high-protein diets the mean counts are very similar in the three diets (6, 7, and 8) and there is no significant effect of the three levels of starch.

Effect of protein.—Where the rations contain no starch (diets 0, 3, and 6) increasing the protein intake has had no significant effect in increasing the density of the ruminal population. The mean counts of these rations show a small increase but the differences are not significant. Where starch is included, however, either at the medium or high level, increasing the protein has resulted in significant increases in the bacterial counts. Thus the mean counts for diets 4 and 5 (47,000,000 and 44,000,000/cu. mm.) are significantly higher than those of diets 1 and 2 (27,000,000 and 21,000,000/cu. mm.) at the 5 per cent. level, and the means of diets 7 and 8 (58,000,000 and 54,000,000/cu. mm.) are significantly higher than those of diets 1 and 2 at the 1 per cent. level. The differences between the mean counts of diets 7 and 8, although higher than those of diets 4 and 5, are not significantly different, indicating that raising the proportion of protein from 9.3 to 11.5 per cent. or from 8.2 to 10.6 per cent. (see Table 2) has not significantly increased the concentrations of the ruminal microorganisms.

(*b*) *Microscopic Characterization of the Microorganisms*

Microscopic characterization of the organisms present in the rumen samples was based on the descriptions by Moir and Masson (1952). A total of 21 group types were identified on the basis of their morphological characteristics in Gram-stained smears. Animals maintained on diet 0 generally showed a high proportion (over 85 per cent.) of Gram-negative organisms, with fewer Gram-positive forms than samples from the sheep fed on the diets containing added protein or starch.

The most notable features of diets 1 and 2 were the increased proportion of Gram-positive organisms and the presence of a pleomorphic Gram-variable rod in long chains clustered about the starch granules. The same organism appeared to a very limited extent in a few animals on diets 7 and 8.

Burroughs *et al.* (1950) have described a similar condition under comparable dietary treatment. Both in their work and in the present experiment, the total bacterial count was very significantly reduced in these cases. Further bacteriological and biochemical work under similar conditions is necessary to assess the biological significance of this relationship.

All other diets produced a high proportion of Gram-positive organisms, more particularly when the total count was low. It is noteworthy that in three of the 12 sheep used, the large *Selenomonas* species, including the organism described by Quin (1943), persisted throughout the experiment on all three dietary treatments and through two treatments in a further two animals. This is difficult to explain, as in preliminary work, when the sheep were maintained on diet 4 in an attempt to standardize the ruminal population, these organisms were eliminated in the rest of the animals within 2 weeks.

(*c*) *Dry Matter Digestibility*

Inspection of Table 3 reveals that the addition of starch, at each level of protein, has brought about little change in the mean dry matter digestibility of the diets. The small differences between diet 0 and diets 1 and 2, between diet 3 and diets 4 and 5, and between diet 6 and diets 7 and 8 are not significant. On the other hand, at all levels of starch feeding the addition of protein has significantly increased the mean dry matter digestibility. Thus the dry matter digestibility of diet 3 (63 per cent.) is significantly higher than that of diet 0 (52 per cent.), of diet 4 (67 per cent.) than that of diet 1 (58 per cent.), and of diet 5 (67 per cent.) than that of diet 2 (55 per cent.), all at the 5 per cent. level. The mean dry matter digestibilities of diets 6, 7, and 8 are significantly higher than those of diets 0, 1, and 2 respectively at the 1 per cent. level. The overall means of dry matter digestibility of the low-, medium-, and high-protein diets are 55, 61, and 71 per cent. These overall means are significantly different.

(*d*) *Nitrogen Balance Data*

In the calculations of the biological values and true digestibility of the nitrogen of the rations a figure of 0.44 g. N per 100 g. food consumed was used for the metabolic faecal N. This is very close to the average figure of 0.5 g. as found by Blaxter and Mitchell (1948), which was found to be somewhat too high for many of our sheep. Endogenous urinary N was assumed to be 0.033 g. N per kg. body weight, as found by Harris and Mitchell (1941).

True digestibility of N.—Inspection of the mean values for the true digestibility of N of the various diets (Table 3) reveals that the proportion of starch in the ration has not influenced these values at any one of the three levels of protein. There is, however, a significant effect of protein at each level of starch. The mean true digestibility of the N of the high-protein diets (6, 7, and 8) is significantly higher, at the 1 per cent. level, than that of the corresponding low-protein diets (0, 1, and 2). The values for the medium-protein diets (3, 4, and 5) are also significantly higher than the corresponding low-protein diets at the 1 per cent. level. The mean true digestibility of the N of the three low-protein diets is 83 and that of the six diets with added protein is 95.

Urinary N as percentage of absorbed N.—The feeding of starch has had no significant effect on these values at any level of protein. Increasing the protein content of the rations from low to medium or from low to high has, however, again had a significant effect at the 1 per cent. level. The percentage of the absorbed N appearing in the urine decreased from an overall mean of 81 in the three low-protein diets to a mean of 40 in the six diets containing added protein. Raising the protein content from medium to high has not significantly altered the position.

Biological values.—The mean figures for the calculated biological values of the N of the various diets (Table 3) indicate an effect of both starch and protein, although the effect of starch is confined to the low-protein diets (0, 1, and 2). The mean of 92 for the medium- and high-starch levels of diets 1 and 2 is significantly higher ($P < 0.01$) than the value of 61 for diet 0. It is obvious, however, that there can be no true assessment of the biological value of the protein of diet 0 because the low protein and available energy of this diet would result in considerable tissue catabolism and because the high rumen population under these conditions has probably resulted also in amino acid deamination with consequent ammonia N loss from the rumen.

No effect of the starch is apparent in the medium- and high-protein diets. The mean biological values of the low- and medium-protein diets are not significantly different but the means of both these diets are highly significantly greater ($P < 0.01$) than that of the high-protein diets. This relatively high level of protein feeding (10.6-13.1 per cent. on the dry basis) has obviously exceeded the maximum required for the full utilization of the absorbed N.

IV. Discussion

It is apparent, from the experiments described, that the concentration of free microorganisms in the rumen is affected by the levels of both protein and starch in the ration. It is apparent, further, that the extent to which either constituent affects this concentration is greatly influenced by the proportion of the other constituent present. Thus, in low-protein diets, increases in the intake of starch result in a marked reduction in the rumen counts accompanied by marked changes in the types of organisms present. Where the diets contain a higher proportion of protein no significant quantitative or qualitative changes in the rumen population occur as the result of increased intakes of starch. Similarly, the effect of protein is influenced by the starch intake. Increases in the level of protein do not significantly alter the rumen counts where the rations are starch-free but increases in protein intake markedly increase these counts where the rations contain medium or high amounts of starch.

It is of interest to compare these findings with those of the earlier experiment of Moir and Williams (1950) in which similar rations were used, except that the protein supplement was casein rather than wheat gluten. It is obvious from Table 4 that, where starch is included in the diets, the values obtained in the two experiments for the concentration of free microorganisms in the rumen are very similar.

Table 4

INFLUENCE OF PROTEIN INTAKE ON THE CONCENTRATION OF RUMINAL BACTERIA

Moir and Williams (1950)				Present Experiments			
Group No.	Starch Intake (g./day)	Protein Intake (g./day)	Rumen Counts (millions/ cu. mm.)	Group No.	Starch Intake (g./day)	Protein Intake (g./day)	Rumen Counts (millions/ cu. mm.)
1	173	15·0	29	1	99	14·2	27
4	99	62·5	55	4	99	63·4	47
5	73	76·9	61	7	99	82·3	58

In view of the relatively constant increase in the numbers of microorganisms with increasing protein intakes that was obtained in the earlier experiment, it was suggested that a constant proportion of the food protein was converted to bacterial protein. This is incorrect on the basis of the *total* protein ingested although, if the concentration of ruminal bacteria is a measure of the proportion of dietary protein converted to bacterial protein, the proportion of *added* protein converted is relatively constant. It is obvious, both in the present and in the earlier experiments, that on this basis the proportion of total dietary protein converted to bacterial protein diminishes sharply as the intake increases. On the same basis the addition of starch to diet 0 has approximately halved this conversion.

The high rumen populations obtained on diets consisting solely of relatively poor roughage are of considerable interest and confirm the findings of Burroughs *et al.* (1950) in this respect. These workers found a high positive correlation between the numbers of ruminal bacteria and the degree of roughage digestion. This is similar to our own data on dry matter digestibility since the addition of protein to starch-containing diets has significantly increased both the mean dry matter digestibility and the mean bacterial density of the rumen. No such correlation exists, however, in the data from diets 0, 1, and 2. The addition of starch has markedly reduced the rumen population without influencing significantly the digestibility of the dry matter. No explanation of these differential effects is yet available although changes in the fixed ruminal flora may be of possible significance. A further problem of great importance raised by these findings is the minimum N requirements of the rumen for bacterial proliferation and function. The high rumen population obtained on the diets consisting only of poor-quality roughage indicates that these requirements are extremely low or at least lower than the minimum requirements of the sheep as a whole for the most effective use of the dry matter and the protein of the diet.

V. Acknowledgments

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INHERITANCE OF SPOTTED WILT RESISTANCE IN THE TOMATO

II. FIVE GENES CONTROLLING SPOTTED WILT RESISTANCE IN FOUR TOMATO TYPES

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Summary

The four spotted-wilt-resistant tomato types, *Lycopersicon pimpinellifolium* and *L. esculentum* varieties Rey de los Tempranos, Pearl Harbour, and Manzana, were each crossed with the susceptible variety Potentate, and also crossed with each other in all possible combinations. The F_1 and F_2 plants, when inoculated with 10 strains of tomato spotted wilt virus, were resistant to five groups of these strains. Three independently inherited recessive genes and two dominant alleles were shown to control resistance to the five groups of spotted wilt strains in the resistant tomato types. Previous evidence of synergism of spotted wilt strains was substantiated by experimental results.

The relative value of each of the four resistant tomato types is discussed together with the best method of utilizing their resistance in practical breeding programmes. A method for selecting resistant phenotypes in hybrid progenies is suggested.

I. INTRODUCTION

Many attempts have been made to breed commercial tomato varieties resistant to tomato spotted wilt (T.S.W.) virus. Porter's strain of *Lycopersicon pimpinellifolium* was used by Kikuta, Hendrix, and Frazier (1945) to breed the variety Pearl Harbour, which was resistant to T.S.W. in Hawaii and appeared to possess a single dominant gene controlling resistance.

Hutton and Peak (1949) used Porter's strain of *L. pimpinellifolium* to breed T.S.W.-resistant hybrids with medium-sized fruit. They reported the inheritance of resistance as being obscure but likely to be controlled by a polygenic system.

Holmes (1948) found that two Argentinian varieties of *L. esculentum*, Rey de los Tempranos and Manzana, were resistant to T.S.W. in New Jersey. Resistance in Rey de los Tempranos appeared to be controlled by a single recessive gene. Holmes concluded that this gene for resistance may be treated as though it were fully recessive in breeding experiments, although F_1 heterozygotes were shown to be more difficult to infect systemically than were the susceptible parent plants. Both Rey de los Tempranos and Manzana were found to be susceptible to T.S.W. in most other areas of the world.

Finlay (1951) reported that the F_1 hybrid of a cross between Pearl Harbour and Rey de los Tempranos had very high field resistance to the disease. The partial resistances of both parents were apparently additive in the F_1 progeny.

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Reporting the results of 12 years of testing resistance of tomato hybrids to natural infection of the T.S.W. virus in field plots, Smith and Gardner (1951) noted that the level of resistance found in Porter's strain of *L. pimpinellifolium* had not been recovered in any of its progeny following a cross with a susceptible variety. No evidence of simple Mendelian inheritance of resistance was obtained.

In all these breeding programmes the resistance or susceptibility of segregating phenotypes was assessed by their reaction to either infection with the disease in the field, or to a natural complex of the virus strains mechanically inoculated. This approach has led to a meagre and confused understanding of the inheritance mechanism of T.S.W. resistance in tomatoes.

Hutton and Peak (1952) have shown that the efficiency of the T.S.W. virus-inactivating system in some resistant tomato species varies considerably with changes in temperature. They suggest that inoculation of hybrid progenies with the ringspot strain of T.S.W. at a constant temperature of 90°F. may facilitate the selection of resistant and susceptible phenotypes.

Finlay (1952) recorded the resistance or susceptibility of some tomato species to 10 strains of the T.S.W. virus under a fixed set of environmental conditions. The present paper records the results of experiments in which the resistance or susceptibility of tomato species and varieties to 10 strains of T.S.W. was used to elucidate the number and mode of inheritance of genes controlling T.S.W. resistance.

II. Materials and Methods

(*a*) *Preparation of Genetic Material*

(i) *Parent Varieties and Species.*—The tomato varieties and species selected for the study of the genetics of T.S.W. resistance were Porter's strain of *L. pimpinellifolium* and *L. esculentum* varieties Rey de los Tempranos, Pearl Harbour, and Manzana. All these types have some form of resistance to T.S.W. The variety Potentate was included as the susceptible control. *L. peruvianum* was excluded from this study because of its cross-sterility with the above-mentioned tomato types.

(ii) *F_1 and F_2 Hybrid Populations.*—The F_1 and F_2 plants required for the first experiments to identify genes for resistance to T.S.W. were obtained by crossing each of the four resistant tomato types with the susceptible variety Potentate. Selfing of some of the F_1 plants produced seed for the F_2 populations.

In the second experiment designed to test allelism, the four resistant types were crossed with each other in all possible combinations. Seed to produce F_1 and F_2 populations was maintained until required.

The parent plants used for these crosses were grown in 4-in. pots in a glass-house. Female flowers were emasculated the day before anthesis and pollen from the male flowers transferred to them the same day.

(iii) *Clones of F_1 and F_2 Plants.*—In order to identify the number of genes present in the resistant varieties, and the T.S.W. strains they controlled, it was desirable to inoculate each of the F_1 and F_2 plants with the 10 strains isolated earlier in this project.

When a number of virus strains are inoculated into a single plant, the interaction and masking effects produced make it impossible to assess the resistance or susceptibility of that plant to any one of the strains.

This problem was overcome by removing 10 shoots from each of the F_1 and F_2 plants and rooting them as separate clones. Each of these 10 genetically identical clones could then be inoculated with a different virus strain. This method was employed throughout this project as a practical means of obtaining the reaction of individual plants to a number of virus strains.

(*b*) *Cultural Techniques*

(i) *Clone Production.*—The F_1 and F_2 plants were grown to a height of about 6 in. in 4-in. pots. The young growing tips were removed to encourage growth of laterals. The growing tips of the laterals were also removed. When there were 10-12 laterals approximately 3 in. long, they were removed and placed in seed boxes of damp sand. The cuttings from each plant were placed in separate rows suitably identified.

The cuttings were kept well watered for 2 weeks, by which time they had rooted and were then transferred into 3-in. pots. The pots were labelled with the original plant number, and a further number from 1 to 10 to facilitate the identification of any particular clone. The whole process from seeds to potted clones was done in an insect-proof glass-house.

(ii) *Experimental Procedures.*—When ready for inoculation, the 10-12 in. clones were transferred to a basement room which was maintained at a constant temperature of 85°F. and illuminated by two banks of fluorescent lights, giving a light intensity of approximately 500 ft.-candles.

The standardization of the inoculum, inoculating technique, and environmental conditions were identical to those described previously (Finlay 1952).

After inoculation all plants were maintained under the fluorescent lights for a period of about 40 days, and were then transferred to an insect-proof glass-house until some fruit had matured in order to check for delayed systemic infection.

The clones of each F_1 or F_2 population to be tested were arranged in a random manner, and inoculated in such a way that every 10 genetically identical clones were inoculated with the 10 virus strains, a different strain for each clone. Each F_1 population was represented by 30 plants. F_2 population size approximated 200 plants in each case. These numbers were multiplied by 10 by the use of 10 clones of each of the original F_1 or F_2 hybrid progeny. 10 parent plants were used in each of the tests for synergistic action between strains of T.S.W.

F_2 segregations were examined by the χ^2 test for goodness to fit to the expected Mendelian ratios.

III. RESULTS

(*a*) *Identification of Genes Controlling Resistance to T.S.W. Virus in Tomatoes*

The F_1 hybrids produced by crossing the four resistant tomato types with the susceptible variety Potentate exhibited three levels of resistance to various strains of T.S.W. (Table 1). Some of the hybrids were resistant, others completely susceptible, and the remainder were susceptible, but delayed systemic infection was a noticeable feature with this class. Although these plants must be classed as susceptible, their resistance to the strains listed is certainly higher than those classed as susceptible. The plants of the control variety Potentate were fully susceptible to all strains.

It will be noted that there is a tendency for resistance to certain strains of the virus to be always associated with other strains, e.g. strains of TB_3, N_1, and R_1 are always found together in the same resistance group. This feature became fully apparent in the F_2 segregations where it was noted that the F_2 plants were resistant to groups of strains. There were five of these strain groups, and each F_2 plant which was resistant to one strain of a group was always resistant to the other strains of the same group. The groups of strains to which the F_2 plants were resistant are shown for each cross in Table 2.

TABLE 1

RESISTANCE IN THE F_1 OF CROSSES BETWEEN SUSCEPTIBLE AND T.S.W.-RESISTANT PARENTS TO 10 STRAINS OF T.S.W. VIRUS

Cross	No. of Plants Inoculated	T.S.W. Strains to Which F_1 Plants are Resistant		
		Resistant	Susceptible—Delayed Systemic Infection	Susceptible
L.p.×S	30	TB_3, N_1, R_1	TB_1, N_2, R_2, R_3, M_1, M_2	TB_2
T×S	30	TB_2	TB_1, N_2, R_2, R_3, M_1, M_2	TB_3, N_1, R_1
PH×S	30	TB_3, N_1, R_1	M_1, M_2	TB_1, TB_2, N_2, R_2, R_3
M×S	30	TB_2	R_2, R_3, M_2	TB_1, TB_3, N_1, N_2, R_1, M_1
S×S	30	—	—	All 10 strains

L.p. = *L. pimpinellifolium*, T = Rey de los Tempranos, PH = Pearl Harbour, M = Manzana, S = susceptible variety Potentate.

The observed segregations were compared with the expected values by means of the χ^2 test. Monogenic inheritance is indicated for T.S.W. resistance to each of the strain groups.

From this evidence it appears logical to assume that five single genes are responsible for plant resistance to each of these strain groups. The resistance gene controlling strains TB_3, N_1, and R_1 is apparently a single dominant, as also is the gene controlling strain TB_2. The rest of the strains come under the control of three recessive genes.

It is suggested that these five genes be given the symbols SW_1^a, SW_1^b, sw_2, sw_3, and sw_4 as shown in Table 3. This table also summarizes the F_1 and F_2 behaviour of these genes. The plant reaction to virus strains M_1 and M_2 is one of immunity. Plants possessing genes SW_1^a, SW_1^b, sw_2, and sw_3 were resistant to the particular T.S.W. strains that these genes control, and gave localized infection following inoculation, but were later able to continue growth free of the disease.

The number of genes for T.S.W. resistance possessed by the five tomato types used in this study varied from none in the fully susceptible variety Potentate to four in both *L. pimpinellifolium* and Rey de los Tempranos (Table 4). No one of these tomato types carried all five genes.

(*b*) *Tests for Allelism*

To discover which, if any, of the five resistance genes were allelic, crosses were made between all the resistant tomato types in all possible combinations and the F$_2$ populations were tested with the 10 T.S.W. strains. The results are recorded in Table 5.

TABLE 2

NUMBERS OF RESISTANT AND SUSCEPTIBLE PLANTS IN THE F$_2$ PROGENY FROM CROSSES BETWEEN SUSCEPTIBLE AND T.S.W.-RESISTANT PARENTS

Cross	Strains to Which Plants are Resistant	No. of Plants Inoculated	Observed No. of Resistant Plants	Expected No. of Resistant Plants	Deviation	χ^2	*P*
L.p.×S	TB$_3$, N$_1$, R$_1$	244	188	183	5	0·520	0·30-0·50
	TB$_1$, N$_2$, R$_2$, R$_3$	244	63	61	2	0·087	0·70-0·80
	R$_2$, R$_3$, M$_2$	244	54	61	7	1·071	0·30-0·50
	M$_1$, M$_2$	244	59	61	2	0·087	0·70-0·80
T×S	TB$_1$, N$_2$, R$_2$, R$_3$	237	66	59·25	6·75	1·024	0·30-0·50
	R$_2$, R$_3$, M$_2$	237	57	59·25	2·25	0·113	0·70-0·80
	M$_1$, M$_2$	237	62	59·25	2·75	0·170	0·50-0·70
	TB$_2$	237	179	177·75	1·25	0·035	0·80-0·90
PH×S	TB$_3$, N$_1$, R$_1$	198	147	148·5	1·5	0·200	0·80-0·90
	M$_1$, M$_2$	198	56	49·5	6·5	1·139	0·20-0·30
M×S	R$_2$, R$_3$, M$_2$	193	41	48·25	7·25	1·453	0·20-0·30
	TB$_2$	193	142	144·75	2·75	0·209	0·50-0·70

L. pimpinellifolium and Pearl Harbour possess the dominant gene SW$_1^a$ controlling resistance to strains TB$_3$, N$_1$, and R$_1$, but they are susceptible to strain TB$_2$. Rey de los Tempranos and Manzana have the dominant gene SW$_1^b$, making them resistant to strain TB$_2$, but they are susceptible to strains TB$_3$, N$_1$, and R$_1$.

It will be seen from Table 5 that, in any cross which brings together these two dominant genes SW$_1^a$ and SW$_1^b$, the F$_2$ populations segregate one plant resistant to strains TB$_3$, N$_1$, and R$_1$, but susceptible to TB$_2$, two plants resistant to TB$_2$, TB$_3$, N$_1$, and R$_1$ and one plant resistant to TB$_2$ but susceptible to strains TB$_3$, N$_1$, and R$_1$. No plants susceptible to all these strains are obtained. These results indicate that SW$_1^a$ and SW$_1^b$ are a single pair of alleles governing the reaction to the two groups of strains TB$_3$, N$_1$, and R$_1$; and TB$_2$. The χ^2 test shows a good fit to a 1:2:1 ratio in the F$_2$ in each case where alleles SW$_1^a$ and SW$_1^b$ are brought together by crossing.

The heterozygous plants, SW_1^a SW_1^b, are resistant to both groups of strains. Under a field epidemic of T.S.W. in which the strains TB_2, TB_3, N_1, and R_1 are present, SW_1^a SW_1^b plants would prove more resistant than either SW_1^a SW_1^a or SW_1^b SW_1^b plants. The recessive genes sw_2, sw_3, and sw_4 appear to be inherited quite independently.

Table 3

INDEX OF GENE SYMBOLS FOR T.S.W. RESISTANCE IN TOMATOES

Gene Symbol	Resistance to T.S.W. Strains	Behaviour in F_1	F_2 Segregation		
			Immune	Resistant	Susceptible
SW_1^a*	TB_3, N_1, R_1	Resistant		3	1
SW_1^b	TB_2	Resistant		3	1
sw_2	TB_1, N_2, R_2, R_3	Susceptible†		1	3
sw_3	R_2, R_3, M_2	Susceptible†		1	3
sw_4	M_1, M_2	Susceptible†	1		3

* Capital letters refer to dominant genes.

† Susceptible but with delayed systemic infection.

The results recorded in Table 5 show the chances of obtaining useful resistance from a breeding programme incorporating any two of the resistant varieties used. The cross between *L. pimpinellifolium* and Rey de los Tempranos produces 50 per cent. of the F_2 plants resistant to all 10 strains of the virus. Only plants having the two dominant alleles SW_1^a, SW_1^b are completely resistant, and, therefore, it is impossible to produce homozygous T.S.W.-resistant plants using these two parents. This also applies to *L. pimpinellifolium* crossed with Manzana, and Rey de los Tempranos crossed with Pearl Harbour. All the other crosses lack one of the genes governing resistance to some of the T.S.W. strains.

Table 4

T.S.W. RESISTANCE GENES PRESENT IN FOUR T.S.W.-RESISTANT TOMATO TYPES

Tomato Type	Genes Present
L. pimpinellifolium	SW_1^a, sw_2, sw_3, sw_4
Rey de los Tempranos	SW_1^b, sw_2, sw_3, sw_4
Pearl Harbour	SW_1^a, sw_4
Manzana	SW_1^b, sw_3

It is apparent from these results that it is impossible to breed a homozygous tomato variety completely resistant to all known strains of T.S.W. by using these four resistant tomato types as parents.

TABLE 5

NUMBERS OF RESISTANT AND SUSCEPTIBLE PLANTS IN THE F_2 PROGENY BETWEEN T.S.W.-RESISTANT PARENTS

Cross	Genes	F_2 Segregations			Observed No. of Resistant Plants	Expected No. of Resistant Plants	Deviation	χ^2	*P*
		Immune	Resistant	Susceptible					
					49	55·75	6·75		
L.p.×T	SW_1^a SW_1^b	1 : 2 : 1*			109	111·50	2·50	1·404	0·30-0·50
(223)†					65	55·75	9·25		
	sw_2	All resistant			—	—	—	—	—
	sw_3	All resistant			—	—	—	—	—
	sw_4	All resistant			—	—	—	—	—
L.p.×PH	SW_1^a	All resistant			—	—	—	—	—
(263)									
	sw_2		1	3	60	65·75	5·75	0·671	0·30-0·50
	sw_3		1	3	62	65·75	3·75	0·285	0·50-0·70
	sw_4	All immune			—	—	—	—	—
					47	49	2		
L.p.×M	SW_1^a SW_1^b	1 : 2 : 1			106	98	8	1·470	0·30-0·50
(196)					43	49	6		
	sw_2		1	3	43	49	6	0·979	0·30-0·50
	sw_3	All resistant			—	—	—	—	—
	sw_4		1	3	45	49	4	0·435	0·50-0·70
					55	54·25	0·75		
T×PH	SW_1^a SW_1^b	1 : 2 : 1			103	108·50	5·50	0·705	0·70-0·80
(217)					59	54·25	4·75		
	sw_2		1	3	60	54·25	5·75	0·812	0·30-0·50
	sw_3		1	3	64	54·25	9·75	2·336	0·10-0·20
	sw_4	All immune			—	—	—	—	—
T×M	SW_1^b	All resistant			—	—	—	—	—
(207)									
	sw_2		1	3	49	51·75	2·75	0·195	0·50-0·70
	sw_3	All resistant			—	—	—	—	—
	sw_4		1	3	56	51·75	4·25	0·465	0·30-0·50
					51	46·75	4·25		
PH×M	SW_1^a SW_1^b	1 : 2 : 1			96	93·50	2·50	1·428	0·30-0·50
(187)					40	46·75	6·75		
	sw_3		1	3	39	46·75	7·75	1·713	0·10-0·20
	sw_4		1	3	46	46·75	0·75	0·016	0·80-0·90

* 1:2:1 Segregation for resistance = 1 resistant (R) to SW_1^a but susceptible (S) to SW_1^b : 2 R to SW_1^a and SW_1^b : 1 R to SW_1^b but S to SW_1^a.

† The number of F_2 plants used in determining the segregations for resistance in each of the crosses.

(c) *Testing for Synergism of T.S.W. Strains*

Some mild and ringspot strains of T.S.W., when associated with a severe strain of the virus, were able to gain access to, and multiply in, host plants which were normally resistant to them in their pure form. This has been cited by Norris (1951) and Finlay (1952) as evidence of synergism.

The partially resistant tomato plants were inoculated with a strain of the virus to which they were susceptible. When infection had become systemic, usually after about 2 weeks, the plants were inoculated with different strains of the virus to which the plants were normally resistant. The results are recorded in Table 6.

TABLE 6

SYNERGISTIC EFFECTS OF T.S.W. STRAINS

Tomato Type	Initial Inoculation*	Plant Reaction to Strains to Which They are Normally Resistant	
		Susceptible	Resistant
Potentate	M_2	TB_2, N_1, R_2	—
L. pimpinellifolium	TB_2	TB_3, N_1, R_2, M_1	—
Pearl Harbour	R_2	N_1	TB_3
	R_2+N_1	TB_3	—
	N_2	TB_3	—
Manzana	M_1	R_2	TB_2
	M_1+R_2	TB_2	—

* The tomato types are fully suceptible to these strains.

Potentate, a susceptible variety, was susceptible to all strains tested. Porter's strain of *L. pimpinellifolium*, infected with strain TB_2, allowed entry and multiplication of a full range of milder strains. It will be seen in Pearl Harbour, which was systemically infected with strain R_2, that infection with a Necrotic strain was necessary before TB_3 could successfully multiply. A similar result was recorded for Manzana infected with M_1, but infection with R_2 did allow entry of strain TB_2.

The mechanism of this reduction in the plant's resistance is not known. The virus-inactivating system in the plant may be inhibited by infection with a tip blight or Necrotic strain, or the by-products of infection from a severe strain supply substances necessary for the successful multiplication of the milder strains which are normally unable to exist in this resistant host plant in pure form. Initial infection with a mild strain allows entry and multiplication of strains in an increasing order of their severity, thus producing a gradual breakdown of the inactivating system over a period of some weeks.

These results substantiate the earlier evidence that a synergism of T.S.W. strains exists in their host plants.

IV. Discussion

During the past three decades, all the breeding programmes designed to incorporate T.S.W. resistance into commercially acceptable tomato varieties have been carried on without adequate knowledge of the inheritance of resistance in T.S.W.-resistant parents. The lack of a satisfactory method of selecting resistant or susceptible phenotypes in hybrid populations is suggested as being the basic cause for this lack of knowledge.

Kikuta, Hendrix, and Frazier (1945) were able to identify a single dominant gene controlling T.S.W. resistance in Pearl Harbour. It is likely that the gene was SW_1, controlling resistance to strains TB_1, N_1, and R_1.

Holmes (1948) indicated the presence of a single recessive gene in Rey de los Tempranos as being responsible for its T.S.W. resistance in New Jersey, where the normal field complex of the virus produced tip blight symptoms on susceptible tomatoes and ringspot symptoms on dahlias. The gene was probably sw_2, controlling strains TB_1, N_2, R_2, and R_3.

These varieties grown in the localities mentioned would not have given a monogenic segregation had there been any other strains of the virus present at the time. If other strains had been present in the local complex these varieties would have either been susceptible, or the added strains would have been controlled by another gene or genes.

From the results recorded in this paper it will be seen that when Pearl Harbour was grown in New Jersey, the major gene necessary to control T.S.W. infection in that area would be absent, thus accounting for the susceptibility of this variety in a different locality.

Workers such as Hutton and Peak (1949) in Canberra, and Smith and Gardner (1951) in California, used Porter's strain of *L. pimpinellifolium* as a source of T.S.W. resistance, in areas where a large number of T.S.W. strains were present in the field complex. They were unable to obtain evidence of simple Mendelian inheritance of resistance.

Hutton and Peak (1952) suggest that the inoculation of hybrid plants at a temperature of 90°F. with the Ringspot strain will facilitate the selection of resistant or susceptible phenotypes. The results contained in this paper indicate that it would be necessary to identify which ringspot strain or strains was being used. The use of ringspot strains would give no indication of the presence or absence of genes sw_4 and SW_1^b. If these genes were absent in the selected phenotypes there could be a disorganization of the plant's virus-inactivating system by a mild strain due to synergistic action.

As there are five genes for resistance to T.S.W., and each gene controls resistance to a group of strains, it is suggested that at least one strain from each group be used in inoculation of clones of hybrid progenies to facilitate the selection of resistant and susceptible phenotypes. Further, it is suggested that the standardized environmental conditions described previously (Finlay 1952) be used, because the results obtained were dependent on this mode of testing.

It is now possible to assess the relative usefulness of these four resistant tomato types as parents in a breeding programme designed to develop spotted-wilt-resistant tomatoes with desirable agronomic characters.

Porter's strain of *L. pimpinellifolium* possesses resistance to all known T.S.W. strains except TB_2, but one or more of the resistance genes are apparently linked with small fruit size and certain other characters of this species.

Hutton and Peak (1952) suggest that Rey de los Tempranos is a better source of resistance than *L. pimpinellifolium* because it has a more efficient inactivating system, has larger fruit, and crosses very readily with commercial varieties. As Rey de los Tempranos carries the same resistance as *L. pimpinellifolium,* except that it has the allele SW_1^b instead of SW_1^a, the suggestion appears to be a useful one.

The occurrence of strain TB_2, which is controlled by allele SW_1^b, has only been recorded in Western Australia (Finlay 1952). For other areas it may be sufficient to replace SW_1^b in Rey de los Tempranos by its allele SW_1^a from Pearl Harbour, and then breed for fruit size and other desirable agronomic characteristics.

Pearl Harbour appears to be useful as a source of the SW_1^a allele, much better than *L. pimpinellifolium* because of its large, high-quality fruit.

Manzana, apart from its large fruit, has little to recommend it as a parent when breeding for T.S.W. resistance, because the two resistance genes it possesses are also to be found as part of the gene complement in Rey de los Tempranos.

Of the four tomato types used in this study, the combination of genes from the varieties Rey de los Tempranos and Pearl Harbour appear to offer the best approach in breeding for T.S.W. resistance, either by the replacement of the SW_1^b allele in Rey de los Tempranos by SW_1^a from Pearl Harbour and breeding a variety homozygous for resistance, or by using the F_1 hybrid produced by crossing these two varieties. If the F_1 hybrid were to be used it would be necessary to increase the fruit size of Rey de los Tempranos before crossing with Pearl Harbour. The delay of systemic infection caused by the action of the heterozygotes of genes sw_2 and sw_3 is probably sufficient to allow a normal crop of fruit to be produced free from symptoms, as reported by Finlay (1951). Increased yield of up to 50 per cent. due to hybrid vigour (Finlay 1951) adds to the benefits to be gained by using the F_1 hybrid of these two varieties.

Complete immunity to all strains of spotted wilt virus could not be obtained from any combination of the four tomato types used in these experiments. Because of its immunity to T.S.W. under field conditions, *L. peruvianum* appears to warrant further study as a parent for use in producing tomato varieties to combat the ever-increasing menace of the spotted wilt virus.

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RELATIVE EFFECTS OF ZINC AND COPPER DEFICIENCIES ON LUCERNE AND SUBTERRANEAN CLOVER

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Summary

The symptoms of zinc deficiency in subterranean clover (var. Dwalganup) and lucerne (var. Hunter River) are described. Bronze spotting was caused by the localized collapse of cells of the upper epidermis. Spherical coacervates which occurred in the underlying cells were different in appearance in the two species. Microchemical tests indicated the presence of phosphorus compounds in the membrane enveloping the coacervates of subterranean clover. The "little leaves" were variable in thickness owing to hypertrophy of the palisade cells. Reduced light delayed the date of appearance of these symptoms.

Copper deficiency produced a marked epinasty of the petioles of the younger leaves of lucerne. Grey to whitish necrotic spots later occurred on the lamina, and death of the top of the plant followed. In subterranean clover, this disorder induced an erect habit of growth and a marginal necrosis of the leaves.

Zinc deficiency caused a significantly greater reduction (1 per cent. level) in the top growth (dry weight) of subterranean clover when compared with lucerne, but there was no significant interaction in the effect of this treatment on roots or the whole plant (tops + roots) between the two species. For both species this treatment significantly reduced top growth more than root growth.

Copper deficiency had a significantly greater effect (1 per cent. level) on lucerne than on subterranean clover for dry weight of tops, roots, and whole plant.

The effect of zinc deficiency was significantly less (1 per cent. level) under reduced light than under full light conditions for both species. The amount of light had no effect on the severity of copper deficiency.

Zinc deficiency decreased the zinc concentration and increased the copper concentration in both lucerne and subterranean clover. However, with lucerne, the zinc concentration was lower, and the coefficient of utilization of zinc was higher, than for subterranean clover, at both normal and deficient levels of zinc in the substrate. For each species, the coefficient of utilization of zinc was higher under conditions of zinc deficiency.

In the control plants the coefficient of utilization of copper was highest in lucerne. Copper deficiency increased the value of this coefficient with subterranean clover, but reduced it in lucerne.

I. INTRODUCTION

It has been reported in the literature that lucerne will make satisfactory growth on certain soils on which other plants show severe symptoms of zinc deficiency. Thus Chandler, Hoagland, and Hibbard (1932); Ballard and Lindler (1934); Hoagland, Chandler, and Hibbard (1936); and Hoagland, Chandler, and Stout (1936) found that lucerne would grow without symptoms on soil in which fruit trees and other plants developed severe symptoms of "little leaf" or zinc deficiency. On soils of the Ninety-mile Plain in South Australia it has similarly been found that, whereas subterranean clover requires a combined dressing of copper and zinc, lucerne will respond to copper, but not to zinc (Riceman 1945, 1948).

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These results suggest either that lucerne had a lower requirement for zinc than certain other types of plants, or that the lucerne roots were able to utilize soil zinc which was unavailable to the roots of the other plants.

Lucerne roots apparently have the property of making the soil zinc more available to other plants. Thus Ballard and Lindler (1934) reported that a cover crop of lucerne materially reduced the amount of "little leaf" in a vineyard so treated. Similarly Hoagland, Chandler, and Stout (1936) found that apricot and corn seedlings grown in pots of "little leaf" soil between lucerne plants established about a year remained almost free of "little leaf" and "white bud" symptoms whereas without the lucerne these symptoms of zinc deficiency were very acute.

In the water culture experiments of Hoagland, Chandler, and Hibbard (1936), the absence of zinc caused "little leaf" symptoms in a range of plants, but no symptoms occurred in the lucerne, although the yield of the control was "better than the minus zinc at one cutting." No yield data were given.

Zinc deficiency symptoms in lucerne grown in sand cultures have recently been described by Hewitt and Bolle-Jones (1951). Their data suggest that lucerne may make relatively better growth under conditions of zinc deficiency than certain other legumes. To date there has been no critical experiment reported in which the relative growth of lucerne and other plants has been studied under known identical conditions of zinc deficiency.

The purpose of the present work was to study the growth of lucerne and subterranean clover in zinc- and copper-deficient water cultures respectively, and thereby obtain data on the relative zinc and copper requirements of the two species. The interaction between light intensity and the effects of zinc or copper deficiency on plant growth was also examined.

II. METHOD

The solution used was that of Arnon (1938). Where possible, solutions of the nutrient salts were eluted several times with a solution of dithizone in carbon tetrachloride to remove heavy metal contaminants. The salts were then recrystallized. The other nutrients to which the dithizone purification technique could not be applied were recrystallized before use.

Double-distilled water produced by a Pyrex glass still was used, and the plants were grown in Pyrex glass beakers blackened on the outside and covered with plaster of paris tops soaked in paraffin. The subterranean clover (var. Dwalganup) and lucerne (var. Hunter River) seed was germinated in acid-washed sand and the plants were transferred to the cultures as soon as they could be conveniently handled. The experiment was commenced on July

3, 1951. In one series of cultures, two clover and two lucerne plants were grown in the same culture pot and in the other series four plants of either clover or lucerne were grown in separate pots. Each treatment in these two series was replicated four times. Half the replicates of each series were placed under full light conditions in the glass-house for the duration of the experiment. The remaining pots were shaded from the direct sunlight and received only approximately 50 per cent. of the illumination of the "full light" series for the first 8 weeks, after which they were transferred to the full light conditions for the remaining 4 weeks of the experiment. The solutions were renewed approximately every 3 weeks.

At the end of the experiment the tops and roots of the plants were harvested separately, oven dried, and weighed.

Histological examinations of certain tissues were made. The material selected was fixed in formalin-acetic-alcohol, dehydrated, embedded in paraffin, and sectioned with a microtome in the usual way. The sections were stained with Connant's Quadruple Combination (Johansen 1940).

III. RESULTS

(*a*) *Symptoms*

The type of symptoms produced in each species by either zinc or copper deficiency was unaffected by growing the two species together or in separate pots, or under full or reduced light conditions. However, the difference in light intensity had a significant effect on the severity of zinc deficiency as described below.

Differences occurred between species with respect to the type of symptom produced by either copper or zinc deficiency.

(i) *Zinc Deficiency*

In both species, the first symptoms appeared approximately 5 weeks after setting up the cultures in full light conditions, but were a fortnight later in the "reduced light" cultures.

Subterranean clover.—The first leaves formed by the plants were normal both as regards size of the leaflets and length of the petioles (Plate 1, Fig. 1). Many of these leaves developed a reddish purple coloration around the midrib at the base of each leaflet. Light brown necrotic spots appeared later, particularly on the distal halves of these leaflets (Plate 1, Fig. 2). These necrotic spots coalesced, and the necrosis extended inwards from the margins of the leaflets. Necrosis of the petioles soon followed. Meanwhile, subsequently formed leaves were slightly chlorotic, and showed a typical "little leaf" condition (Plate 1, Figs. 1 and 3). The size of the leaflets and the length of the petioles were very reduced and the length of the internodes between leaves was shortened, giving the plants a rosetted appearance.

The "little" leaves were usually narrower in proportion to their length than normal leaflets, and many were deeply notched at the apex (Plate 1, Fig. 3).

They did not develop any necrotic spots as described above for the first-formed leaves. However, many of them showed a reddish purple coloration, together with localized chlorotic areas towards the margins, and visible in the upper surface.

A histological examination of extremely dwarfed little leaves was made. It was found that the thickness of the leaves was very variable — marked thickening occurred in places, particularly towards the edges (Plate 1, Fig. 4). Here the thickness of the little leaves was actually greater than that of normal leaves, whereas the thin portions of the former were of normal thickness. The little leaves were usually folded upwards to form a deep crease at the midrib (Plate 1, Fig. 4).

Severe disorganization of the tissues themselves was evident. The upper epidermis was uneven in appearance, and there were marked projections on many of the cells (Plate 2, Fig. 2). However, the palisade cells were the most seriously affected. In many cases they were divided to form rhomboidal rather than columnar-shaped cells. In the thin portions of the leaf, this cell division led to the virtual disappearance of the palisade layer, as its cells were little different in size and shape to the underlying spongy-mesophyll cells (Plate 2, Fig. 2). The thickening of portions of the little leaves was caused by a more extensive hypertrophy of the palisade cells. Several layers of these cells occurred, and many retained the normal columnar shape (Plate 2, Figs. 3 and 4).

Some of the plastids showed elongation. However, in localized areas, lytic factors had destroyed many of the plastids. In some cells the contents had been converted into materials which could not be detected microscopically (Plate 2, Fig. 4). This lysis of the plastids was correlated with the development of the reddish purple coloration or localized chlorosis referred to above.

Cell necrosis first occurred in the mesophyll cells. Identical manifestations of cellular disorganization in "little" leaves of various plants have been described by Reed (1938, 1939) and Reed and Dufrenoy (1935).

The cytology of the first-formed normal-sized leaves of the zinc-deficient Dwalganup plants was very different from that of the "little" leaves of the same plants as described above. In contrast with the latter, these first-formed leaves were normal with respect to the development of the epidermal, palisade, and spongy mesophyll layers. However, the tissues were abnormal in that many of the cells were devoid of plastids, and contained large, usually spherical bodies evidently identical in nature with the coacervates described as occurring in the cells of zinc-deficient plants by Reed and Dufrenoy (1942). Although they were most common in the palisade cells, the coacervates occurred also in the epidermal and mesophyll cells of the zinc-deficient subterranean clover leaflets (Plate 3, Figs. 1 and 2). The tissue towards the edge of the leaflet contained more coacervates than that near the midrib. The coacervation of the cell contents evidently preceded the extension of the marginal necrosis of the affected leaves as described above.

The phosphoric acid test, using the molybdenic reagent described by Reed and Dufrenoy (1942), was applied to sections of the clover leaflets. The result of the test was similar to that obtained by these workers, as the membrane enveloping the coacervate was stained blue, indicating the presence of phospholipoids.

The first signs of the necrotic spotting of the oldest, normal-sized leaves of the zinc-deficient subterranean clover plants (Plate 1, Fig. 2) consisted of a localized collapse of the upper epidermis (Plate 3, Figs. 1 and 2). Later the underlying tissues were also involved. This phenomenon of upper epidermal collapse is identical with that associated with the occurrence of bronze spotting of zinc-deficient flax leaves (Millikan 1951).

Lucerne.—The first symptoms consisted of bronze-coloured spots around the distal margins of the upper (but not the youngest) leaves of the plant (Plate 4, Fig. 1). These spots first occurred on the upper surface of the leaf, but later they could also be seen from the under surface. Subsequently the bronzed areas became white and necrotic. At a later stage some leaves showed bronze spots which were scattered in a random fashion over the surface of the leaflets rather than concentrated near the distal margins (Plate 4, Fig. 2).

Meanwhile, the rate of growth was retarded, tillering was reduced, and the whole plant assumed a slight chlorotic appearance. The younger leaves were smaller and much stiffer than normal and their margins were inrolled (Plate 4, Fig. 4). Reduction in leaf size was particularly severe in the tillers produced at the base of the plant (Plate 4, Fig. 4). Many had a deep notch at the apex (Plate 4, Fig. 3), and some showed a chlorotic mottling. They also had a leathery feel. The middle leaves of the plants finally developed a grey necrosis which started at the tips and extended down the lamina until the whole leaf and the top of the petiole was involved. There was a marked tendency for these affected leaves to fall from the plant.

A histological examination of various tissues from the zinc-deficient lucerne plants revealed similar cellular changes to those described above for subterranean clover. The normal lucerne leaf is characterized by a well-developed palisade player with long, columnar-shaped cells and large intercellular spaces (Plate 5, Fig. 5).

The first signs of the bronze spotting referred to above consisted of a collapse of the cells of the upper epidermis (Plate 5, Fig. 1). The underlying tissues appeared normal at first, but the cells soon showed signs of disorganization. The cell contents disintegrated and became necrotic. This sequence of cellular changes was identical with that associated with the bronze spotting of zinc-deficient subterranean clover leaves.

Small, spherical bodies with a marked affinity for safranin sometimes occurred in the zinc-deficient lucerne tissues directly associated with the development of a bronze necrotic spot (Plate 5, Fig. 3). These bodies did not occur in the cells of normal appearance in other parts of the leaf, nor did they occur in every lesion examined. They were apparently similar in nature to the coacervates in zinc-deficient subterranean clover tissue, but were smaller and stained differently to the latter. However, the upper epidermal cells of the lucerne leaves occasionally contained larger coacervates (Plate 5, Fig. 4).

In contrast with those of subterranean clover, the little leaves of lucerne were not variable in thickness, although they did show similar cellular changes.

Subdivision of the palisade cells, and the reduction in the intercellular spaces occurred (Plate 5, Fig. 6). This latter change probably accounted for the stiffer and leathery feel of the little leaves when compared with normal leaves.

(ii) *Copper Deficiency*

Symptoms first occurred on the same day both in the full light and reduced light cultures. This was approximately 5 weeks after the commencement of the experiment.

Subterranean clover.—Growth was retarded and the plants became slightly chlorotic and developed a habit of growth more erect than normal (Plate 3, Fig. 3). This erect habit of growth was apparent before any necrotic leaf symptoms developed. Leaf size was unaffected, but the edges of the younger leaflets commenced to roll upwards and inwards. Later the inrolled margin became necrotic, while the remainder of the lamina was distorted and puckered (Plate 3, Fig. 4). This marginal necrosis commenced in any place on the edge from the centre to the tip of the leaflet, and varied in colour from white or light brown to bluish green. In the latter case, the margin between necrotic and unaffected tissue was usually a light brown colour. The necrosis later involved the whole of the leaf and petiole. Many of the older leaves developed pinkish cinnamon-coloured necrotic spots approximately 1-2 mm. in diameter. No flowers were produced in the absence of copper, whereas the controls flowered freely.

Piper (1942) reports a fourfold reduction in dry weight of subterranean clover grown in copper-deficient solutions when compared with the yield of plants grown in solutions containing an adequate supply of copper. In the absence of copper his plants were at first slightly chlorotic, but he states that many of them later withered and died without developing any other symptoms. However, copper deficiency in alsike clover has been described by Hewitt and Bolle-Jones (1950). The symptoms of marginal cupping and necrosis of the leaflets were similar to the subterranean clover symptoms described in this paper. Rossiter (1951) observed less severe symptoms in Dwalganup subterranean clover plants grown in copper-deficient soil, which were similar in many respects to those outlined above.

Lucerne.—The first symptom consisted of a marked epinasty of the petioles of the younger leaves (Plate 6, Fig. 1), the terminal leaflets being bent right back against the petioles. Light grey to white necrotic spots soon appeared on these leaves, which were then slightly chlorotic. The spots sometimes appeared on the leaf margins, but most characteristically they occurred towards the base of the leaflets as shown in Plate 6, Figure 2. However, the necrosis soon involved the whole of the leaflets and petiole. The size of the youngest leaflets was very much reduced and death of the top of the plant followed (Plate 6, Fig. 3). Piper (1942) has also described symptoms of epinasty in the petioles of lucerne plants grown in copper-deficient water cultures.

(*b*) *Relative Effects on Growth*

The dry weights of the plants grown in these cultures are presented in Tables 1 and 2. A general view of portion of the experiment is shown in Plate 7. As the species grown in different pots had a larger error variance than those grown together in the same pot, it was necessary to use weights proportional to the inverse of the variances for statistical purposes. The transformed values given in Tables 1 and 2 are derived from the weighted means of the two sets of data.

TABLE 1

MEAN DRY WEIGHT (G. PER PLANT) OF SUBTERRANEAN CLOVER (VAR. DWALGANUP) AND LUCERNE (VAR. HUNTER RIVER) GROWN IN NUTRIENT SOLUTIONS

Transformed values* in parentheses

Treatment	Subterranean Clover			Lucerne		
	Tops	Roots	Tops+Roots	Tops	Roots	Tops+Roots
Complete solution	3·631 (3·491)	0·434 (2·630)	4·065 (6·121)	3·587 (3·541)	1·575 (3·192)	5·162 (6·733)
Zinc deficiency	0·356 (2·530)	0·160 (2·195)	0·516 (4·725)	0·891 (2·892)	0·491 (2·663)	1·382 (5·555)
Copper deficiency	0·534 (2·713)	0·111 (2·035)	0·645 (4·748)	0·333 (2·446)	0·118 (1·947)	0·451 (4·393)

Significant differences: interaction effect (transformed values)	5 per cent. Level	1 per cent. Level
Within species	0·109	0·148
Between species (tops or roots)	0·198	0·269
Between species (tops + roots)	0·350	0·490

* Log_{10} $(X \times 10^3)$ of weighted means.

A comparison of the results obtained from the series where the two species were grown in separate pots and that where they were grown together in the same pot revealed no significant difference in dry weight, nor did these two methods of culture have any significant interaction effects with zinc or copper deficiencies, or with the amount of light. Because of this negative result these figures are not included in this paper.

Zinc deficiency caused a significantly greater reduction in dry weight of the tops of subterranean clover than of lucerne, but the zinc species interaction was not significant either with respect to roots or roots + tops.

By contrast, copper deficiency had a significantly greater effect on lucerne than on subterranean clover for dry weight of tops, roots, and tops + roots.

As regards the effect on plant parts the results show that zinc deficiency caused a significantly greater reduction in top growth than root growth for both subterranean clover and lucerne.

The effect of copper deficiency on plant parts was not consistent for the two species. In subterranean clover the top growth was reduced by this treatment to a greater degree than root growth, whereas with lucerne root growth was affected more than top growth.

The interaction between light intensity and zinc deficiency was highly significant (Table 2), i.e. the reduction in growth due to the absence of zinc was less under reduced light than under full light conditions. The amount of light had no significant effect on the severity of copper deficiency symptoms.

TABLE 2

EFFECT OF LIGHT INTENSITY ON ZINC AND COPPER DEFICIENCY. COMBINED RESULTS FOR SUBTERRANEAN CLOVER AND LUCERNE (G. DRY WT. PER PLANT)

Transformed values* in parentheses

Treatment	Full Light	Reduced Light
	Dry Wt. (g.)	Dry Wt. (g.)
Complete solution	6·094 (3·325)	3·130 (3·052)
Zinc deficiency	0·828 (2·547)	1·051 (2·593)
Copper deficiency	0·691 (2·382)	0·405 (2·188)

	5 per cent. Level	1 per cent. Level
Significant difference: interaction effect (transformed values)	0·175	0·245

* Log_{10} $(X \times 10^3)$ of weighted means. The results of the two species are combined in this table as the statistical analysis showed that there was no significant interaction between amount of light and either species or plant part.

(c) *Utilization of Zinc and Copper*

The results of copper and zinc analyses of plants from the series where the two species were grown together in the same pot are presented in Table 3.

There was a decrease in zinc concentration and the absolute amount of zinc in the tops, a marked increase in copper concentration, and a decrease in the Zn/Cu ratio under conditions of zinc deficiency in both subterranean clover and lucerne. On the other hand, the copper concentration of the copper-deficient plants was only slightly less than normal for subterranean clover and was actually higher for lucerne. However, the Zn/Cu ratio was comparable to that of the control treatment, while the total copper content in the tops was much reduced for both species. Piper (1942) has reported that the copper concentration in the tops of Algerian oats grown in a nutrient solution without the addition of copper was higher than that of plants grown in other cultures to which small amounts of copper had been added. The possible significance of this result has been discussed by Ulrich (1952).

Table 3

COPPER AND ZINC CONTENTS (ON DRY BASIS) OF TOPS OF SUBTERRANEAN CLOVER (VAR. DWALGANUP) AND LUCERNE (VAR. HUNTER RIVER)* SHOWING THE UTILIZATION OF THESE TWO ELEMENTS

Nutrient Solution	Light Conditions	Subterranean Clover								Lucerne							
		Top Dry Wt. per Plant (g.)	Copper (Cu) (p.p.m.)	Zinc (Zn) (p.p.m.)	Ratio Zn/Cu	Cu per Plant (μg.)	Zn per Plant (μg.)	Coefficient of Utilization†		Top Dry Wt. per Plant (g.)	Copper (Cu) (p.p.m.)	Zinc (Zn) (p.p.m.)	Ratio Zn/Cu	Cu per Plant (μg.)	Zn per Plant (μg.)	Coefficient of Utilization*	
								Cu	Zn							Cu	Zn
Complete solution	Full	4·814	6·2	76	12·3	29·8	366	0·162	0·013	5·415	3·9	48	12·3	21·1	260	0·257	0·021
	Reduced	1·895	6·5	90	13·8	12·3	171	0·154	0·011	2·365	4·4	39	8·9	10·4	92	0·228	0·026
Zinc deficiency	Full	0·320	25	25	1·0	8·0	8·0	—	0·040	0·762	10·8	13	1·2	8·2	9·9	—	0·077
	Reduced	0·336	27	24	0·9	9·1	8·1	—	0·042	0·869	10·4	13	1·3	9·4	11·3	—	0·077
Copper deficiency	Full	0·602	4·8	62	12·9	2·9	37·3	0·208	—	0·338	5·2	69	13·3	1·8	23·3	0·188	—
	Reduced	0·465	5·1	87	17·1	2·4	40·5	0·194	—	0·211	‡	74	—	—	15·6	—	—

* The plants are from the series where the two species were grown together in the same pots (see Plate 7).

† Dry matter produced (g.)/zinc or copper absorbed (μg.).

‡ Sample lost.

The zinc concentration of lucerne was less than that of subterranean clover except under copper deficiency where the figures were of comparable magnitude. Conversely, the coefficient of utilization of zinc (i.e. dry matter produced (g.)/zinc absorbed (μg.)) of both the normal and zinc-deficient lucerne was higher than for subterranean clover. These data indicate that the zinc requirement of lucerne was less than that of the subterranean clover and support the conclusions drawn from the data in Table 1.

IV. Discussion

The experiment described above has demonstrated the existence of highly significant differences in the requirements of zinc and copper between the two species studied. Lucerne grew better than subterranean clover under conditions of zinc deficiency, whereas subterranean clover grew better than lucerne when copper was deficient in the substrate.

The author (unpublished data) has shown that the Dwalganup variety of subterranean clover (which was used in the present experiment) is less affected by zinc deficiency than the later varieties Mt. Barker and Bacchus Marsh. Thus, if either of these two varieties had been used in the present experiment, the difference in the effect of zinc deficiency between subterranean clover and lucerne would doubtless have been greater.

There was no significant difference between the results obtained from cultures where the two species were grown together in the same pot and those where they were grown in separate pots. Thus there is no evidence that either of the two species, when grown together, utilized any limited supply of zinc or copper available to them in the deficient cultures at the expense of the other species.

Zinc deficiency caused a relatively greater reduction in top growth than in root growth of both species. A similar interaction between plant part and zinc deficiency in subterranean clover was obtained in previous experiments by Millikan (unpublished data). Wood and Sibly (1950) found that a greater percentage of the total zinc in the plant was contained in the roots of oats than in the other organs of this plant. The relatively greater effect of zinc deficiency on top as compared with root growth of lucerne and subterranean clover could, therefore, be attributed to the accumulation in the roots at the expense of the tops of the very limited amount of zinc available to the plants.

Although lucerne made better growth than subterranean clover in the zinc-deficient water cultures, the former species still showed very severe symptoms of zinc deficiency, indicating that an adequate supply of zinc in the substrate was essential for its normal development. However, the literature citations given in the introduction to this paper show that lucerne fails to develop zinc deficiency symptoms, or respond to zinc, when grown on soils on which other plants show severe symptoms, and also that the proximity of lucerne roots will cure zinc deficiency symptoms in more susceptible plants. The better utilization by lucerne of the zinc supply, as demonstrated in the present experiment, would only partly account for these phenomena. It is suggested that excretions from lucerne roots have the property of increasing the availability of soil zinc not only for their own use, but for the roots of other plants. The deep penetration of lucerne roots, which may perhaps enable them to exploit soil zinc outside the range of shallower-rooted species, does not affect this conclusion. This is indicated by the work of Hoagland, Chandler, and Stout (1936) cited earlier, where the roots of the lucerne and other species were confined to the same pot.

In the present experiment the growth of subterranean clover was significantly better than that of lucerne under identical conditions of copper deficiency, irrespective of whether the two species were grown together in the same pot or in different pots. This accords with the reports of Trumble and Ferres (1946) and Hewitt and Bolle-Jones (1951) that lucerne was the most responsive to copper of the legumes (including subterranean clover) which they grew in copper-deficient substrates. The results given in Table 3 indicate a difference in the capacities of lucerne and subterranean clover to utilize absorbed copper under conditions of copper deficiency rather than to differences in their absorptive capacity for copper.

However, differences in the absorptive capacity of plant species for copper have been reported. Rye is much more resistant than oats to copper deficiency (Piper 1938, 1942; Riceman 1943), while resistant species and varieties of oats occur (Rademacher 1937; Riceman 1943). In contrast with the present experiment, these differences have not been explained by differences in copper requirement as the resistant plants were able to extract more copper than susceptible plants from the copper-deficient soils (Rademacher 1937; Piper 1942). On normal soils there was no difference in copper values between resistant and susceptible varieties.

The increase in the severity of zinc deficiency symptoms with increasing light intensity demonstrated in this paper accords with the results of previous investigators (Haas 1936; Chapman, Vanselow, and Liebig 1937; Reed 1938; Hoagland 1944). Skoog (1940) further showed that zinc-deficient plants maintained a high auxin content and continued to elongate in red or weak light, but in blue light an early cessation of growth and reduction in auxin content occurred.

Differences in light duration, as distinct from light intensity, may also affect the plant's capacity to utilize zinc from a relatively unavailable source. In this regard Trumble and Ferres (1946) showed that on a zinc-deficient soil, the growth of subterranean clover (variety Mt. Barker) was significantly increased by increased light duration.

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EXPLANATION OF PLATES 1-7

PLATE 1

Fig. 1.—Zinc-deficient subterranean clover plants. The first leaves are normal in size, but leaves produced later show a marked reduction in size of lamina and length of petioles.

Fig. 2.—Necrotic spotting on the upper surfaces of oldest leaves of zinc-deficient subterranean clover.

Fig. 3.—Enlarged views of very dwarfed "little" leaves showing their abnormal shape.

Fig. 4.—Transverse section through a "little" leaf. The thickness of the leaf is very variable.

PLATE 2

Sections through subterranean clover leaves.

Fig. 1.—Leaf of normal plant grown in full nutrient solution.

Figs. 2-4.—"Little" leaves from zinc-deficient plants similar to those shown in Plate 1, Figures 3 and 4. Cellular disorganization in the little leaves is apparent. Projections from the epidermal cells occur. In Figure 2 the cells of the palisade layer are little different from those of the mesophyll layer as the former are rhomboidal rather than columnar in shape. The increased thickness in places of the little leaves (see Plate 1, Fig. 4) is due to the marked hypertrophy of the palisade layer (Fig. 3), which consists of several layers of more or less columnar-shaped cells. Lytic factors have destroyed the contents of the cells of the hypertrophied palisade layer in Figure 4. This lysis was associated with the occurrence of a chlorotic area in the leaf.

E, epidermis; *P*, palisade layer; *M*, mesophyll.

PLATE 3

Figs. 1 and 2.—Sections through normal-sized leaves of zinc-deficient subterranean clover similar to those shown in Plate 1, Figures 1 and 2. Necrotic spotting (Plate 1, Fig. 2) commences as a localized collapse of the cells of the upper epidermis (*E*). Large coacervates (*C*) occur in the cells of both the palisade and mesophyll layers.

Fig. 3.—Subterranean clover plants growing in full nutrient solution (left) and copper-deficient solution (right). The latter show abnormally erect habit of growth.

Fig. 4.—Younger leaves of copper-deficient subterranean clover showing marginal necrosis and cupping.

PLATE 4

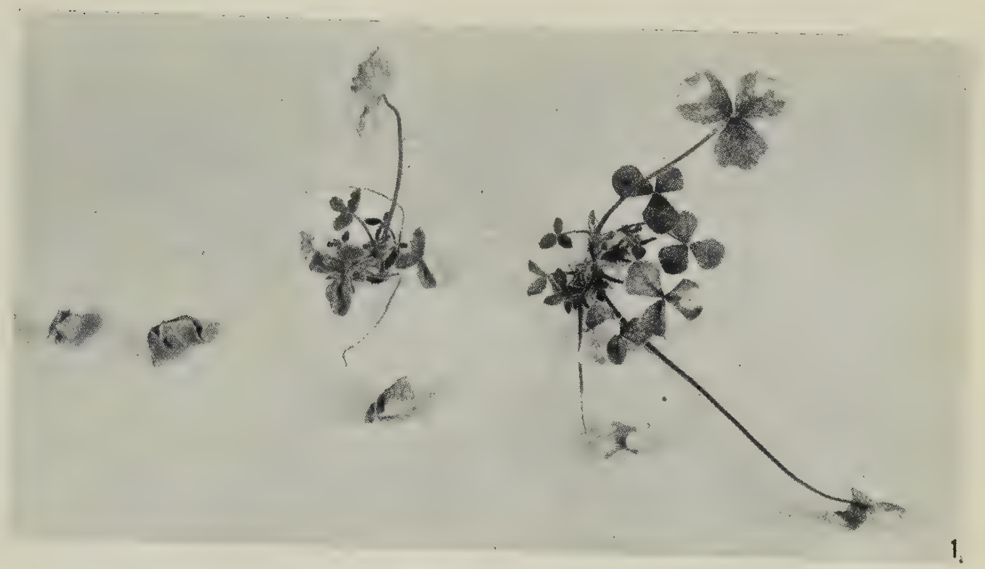
Fig. 1.—Middle leaves from lucerne plant grown in a zinc-deficient nutrient solution. The marginal spots are bronze-coloured at first, but later develop a white centre (right).

Fig. 2.—Zinc-deficient lucerne leaves showing numerous bronze spots on the upper surface.

Fig. 3.—Left, normal leaf. Right, "little" leaves from base of zinc-deficient lucerne plant, showing their abnormal shape.

Fig. 4.—Severe reduction in size of leaves of secondary shoots from the base of a zinc-deficient lucerne plant. The leaves at the top of the plant were also smaller and much stiffer than normal, and their margins were inrolled upwards.

PLATE 1



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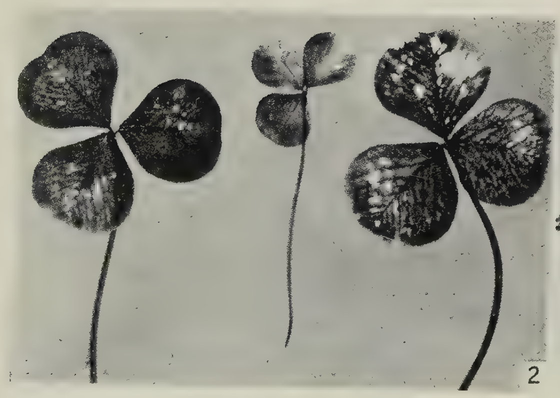

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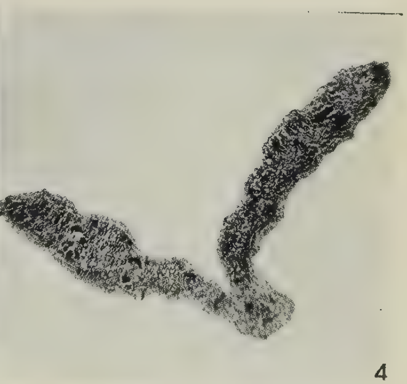

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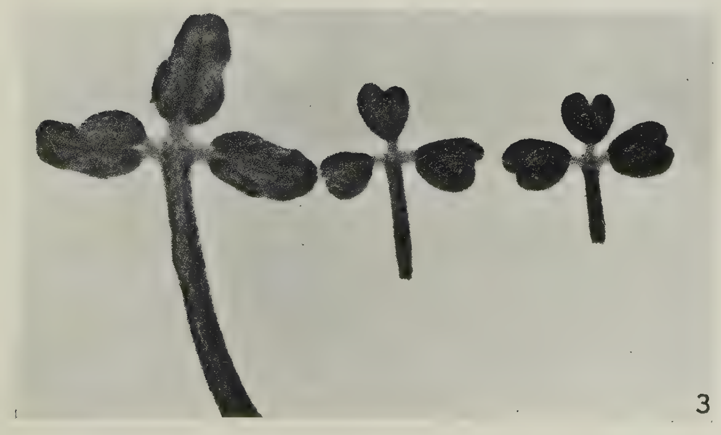

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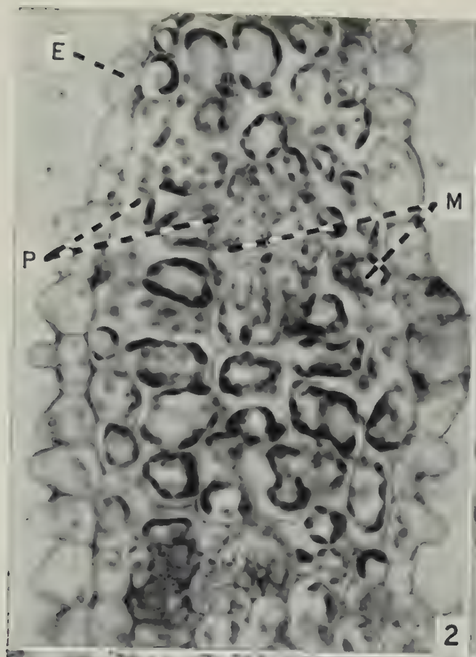
Zn AND Cu DEFICIENCIES ON LUCERNE AND SUBTERRANEAN CLOVER



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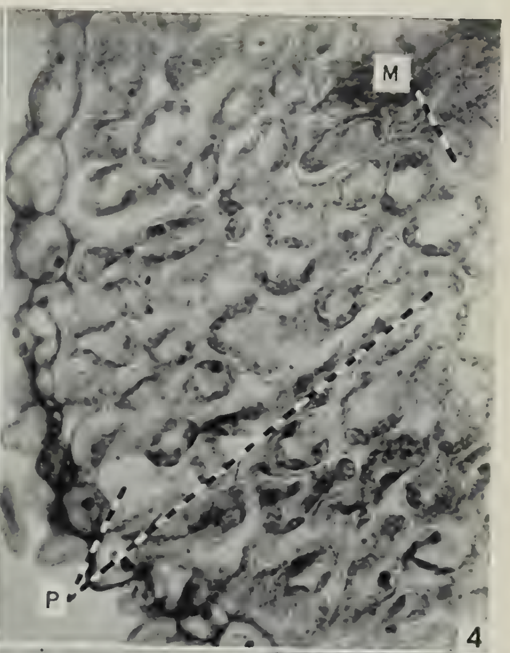

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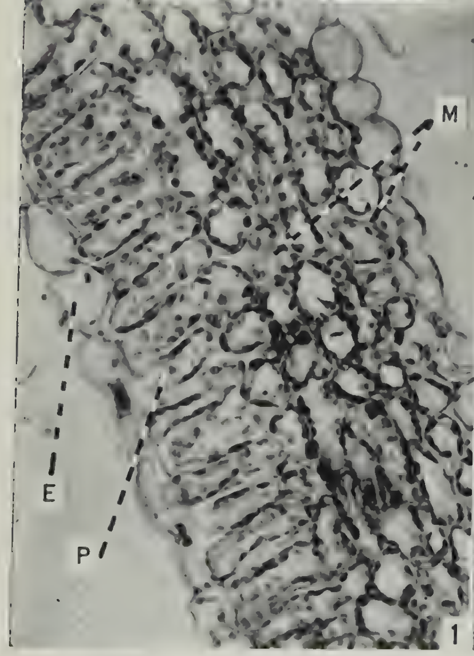

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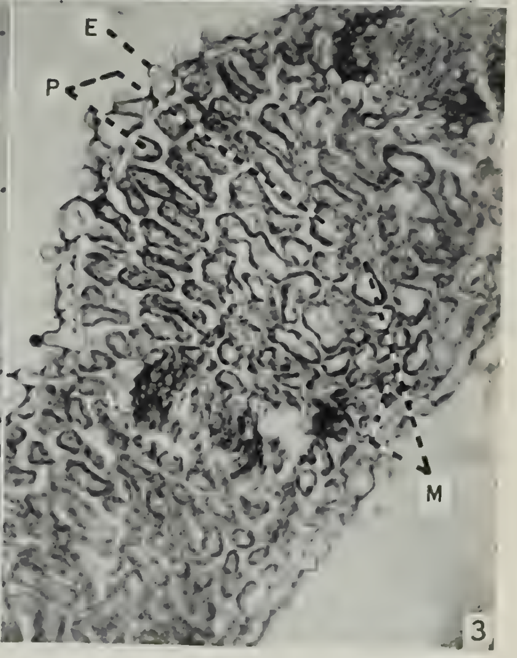

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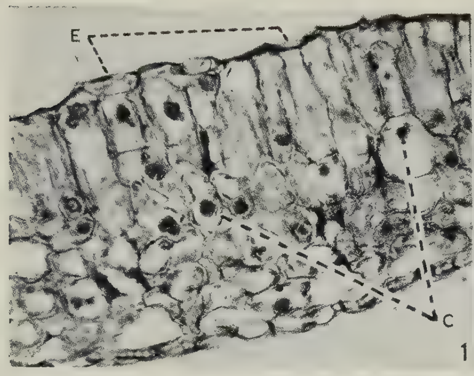

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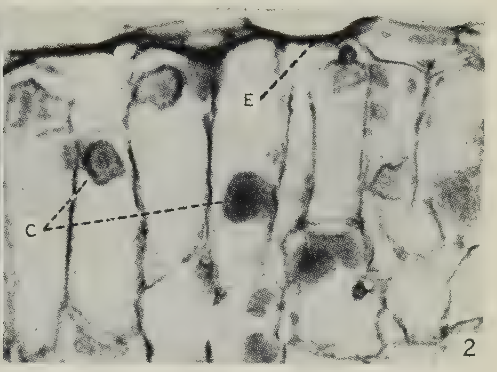

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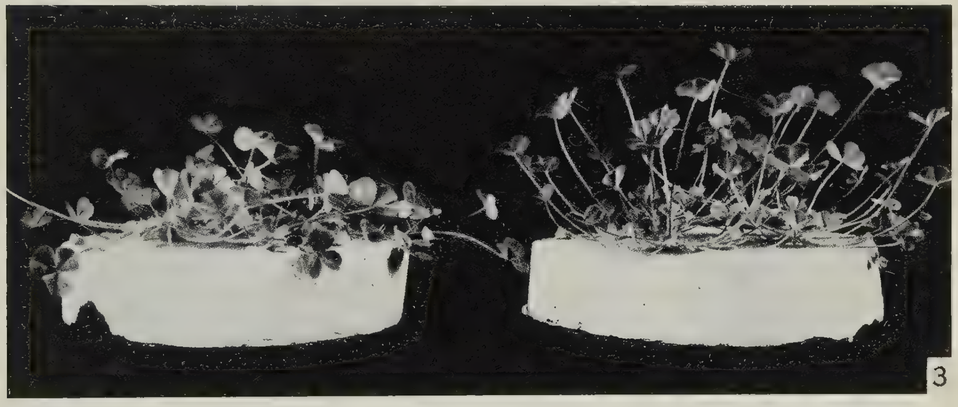

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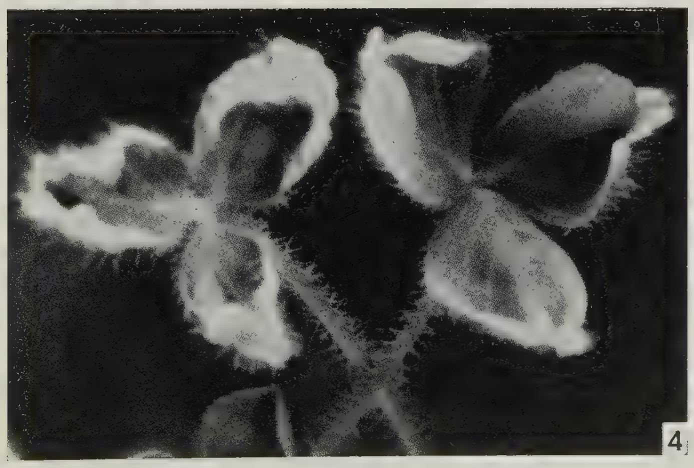

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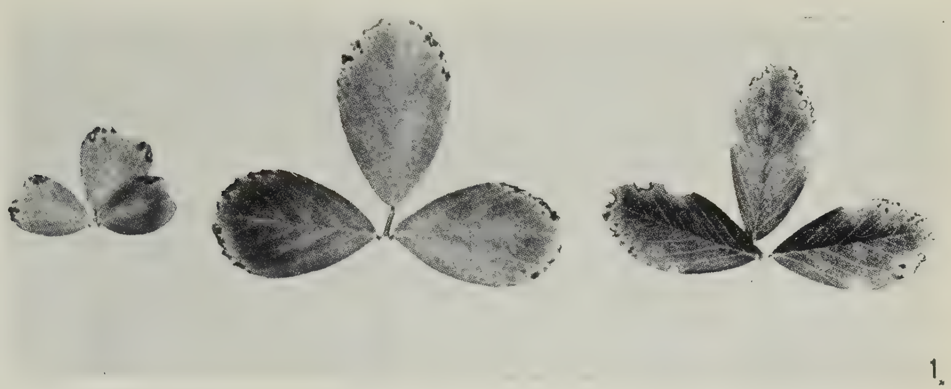

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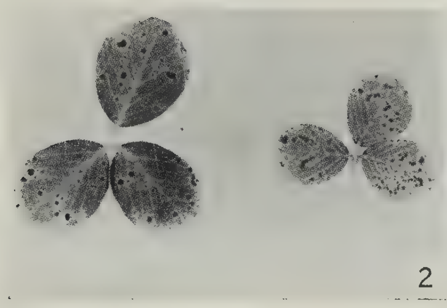

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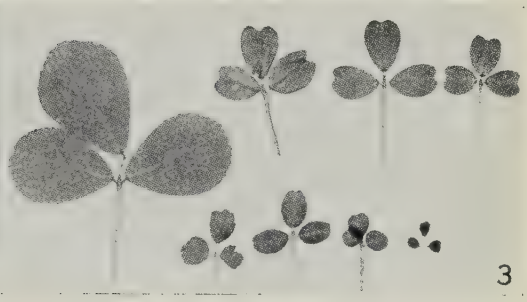

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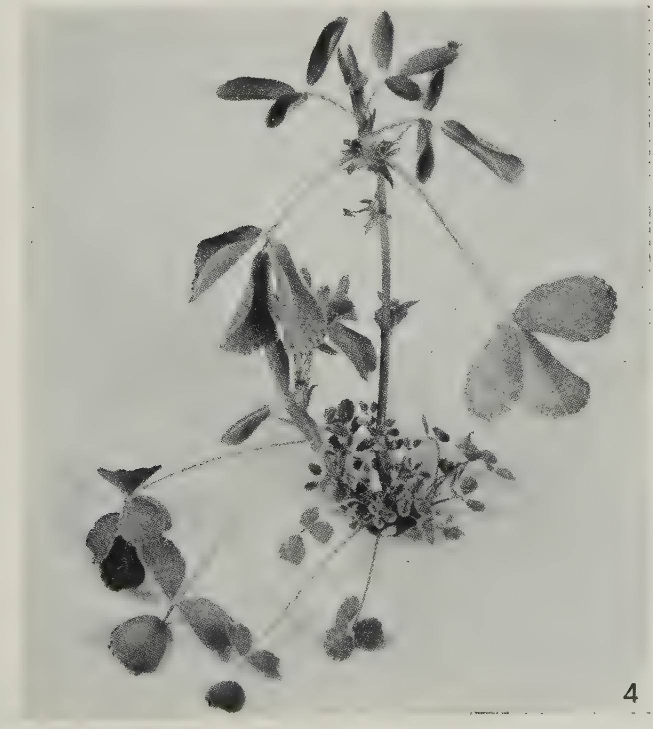

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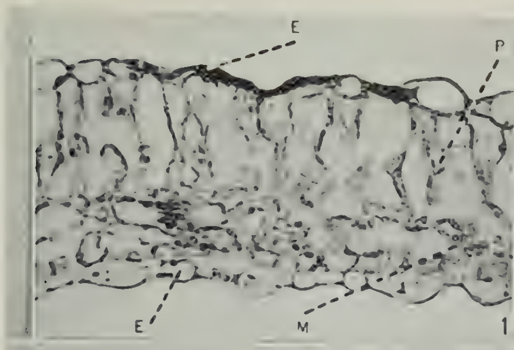

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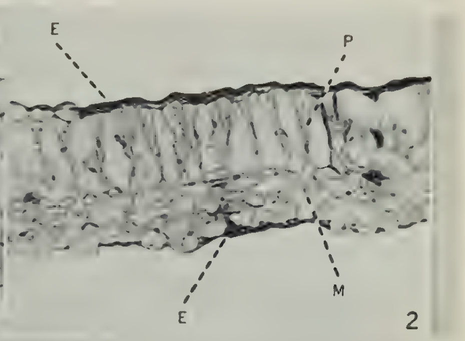

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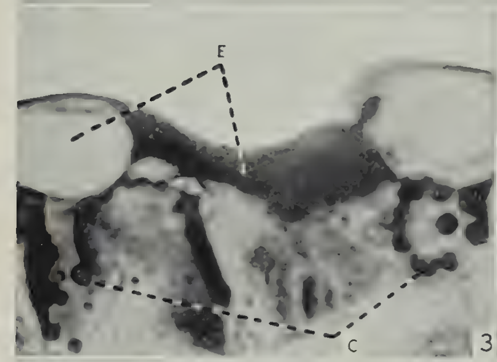

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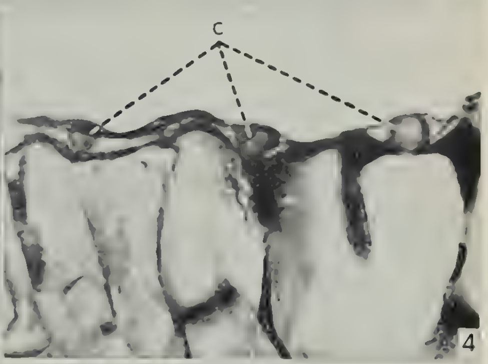

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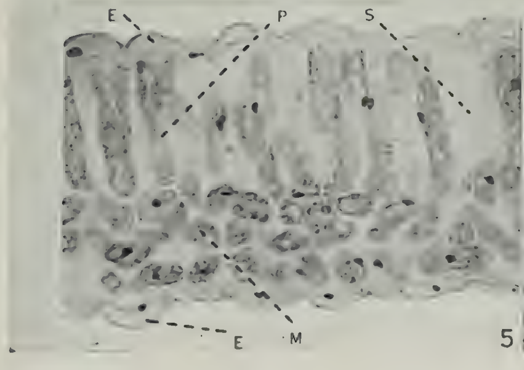

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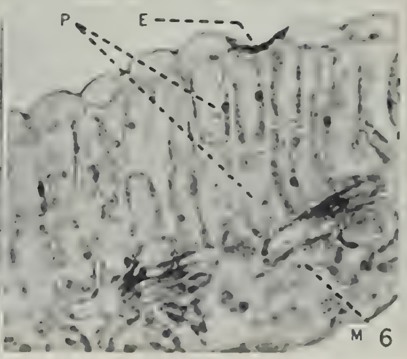

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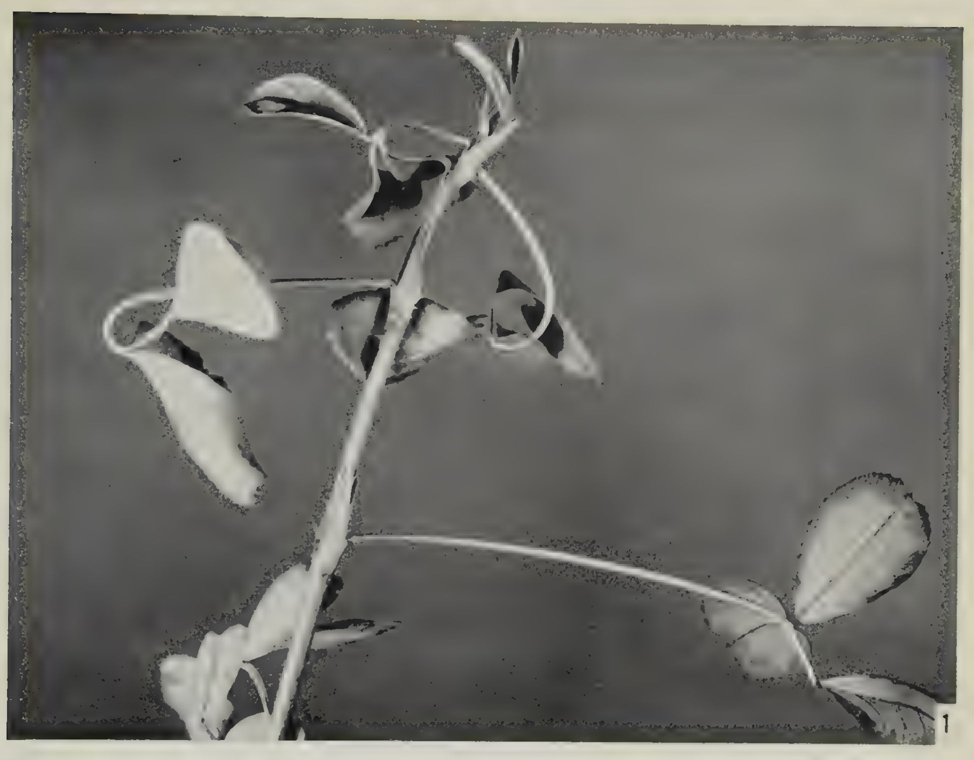
Zn AND Cu DEFICIENCIES ON LUCERNE AND SUBTERRANEAN CLOVER



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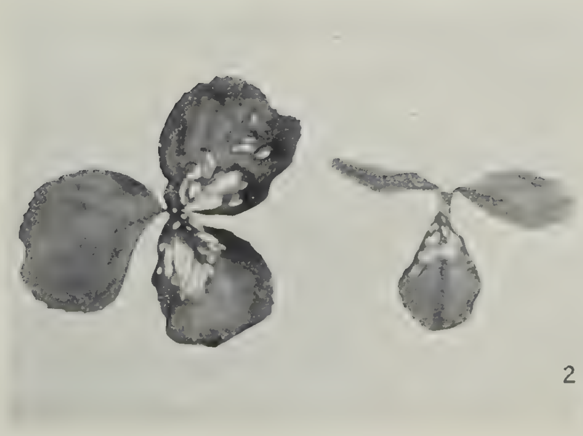

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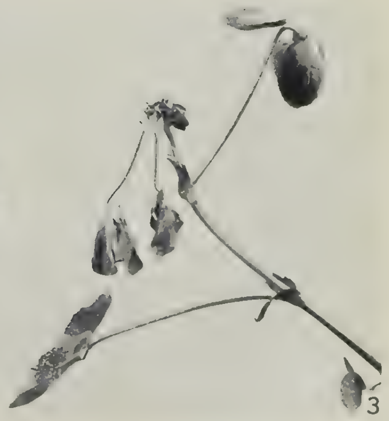

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Zn AND Cu DEFICIENCIES ON LUCERNE AND SUBTERRANEAN CLOVER

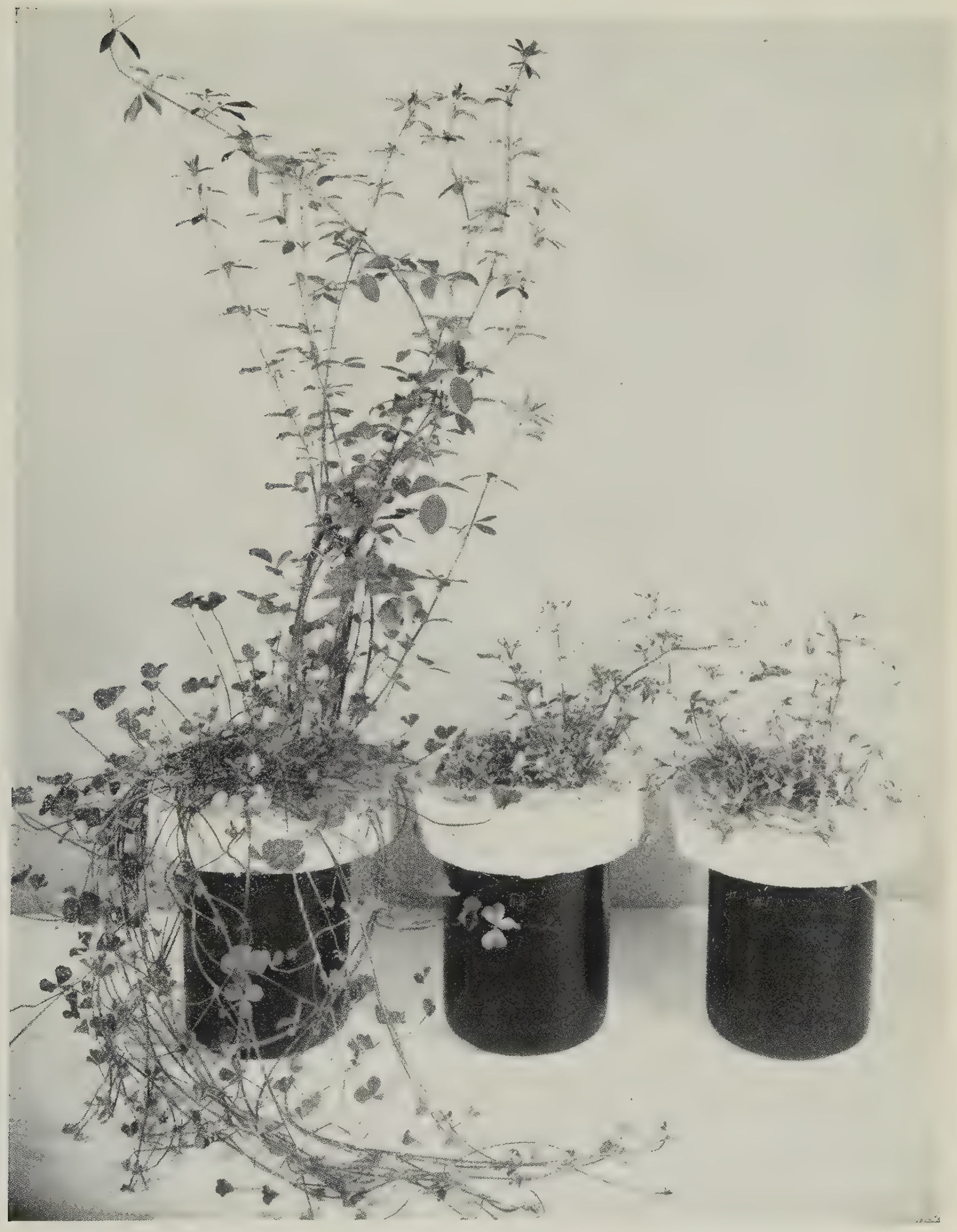

PLATE 5

Figs. 1 and 2.—Sections through bronze spots on zinc-deficient lucerne leaves, similar to those shown in Plate 4, Figure 2. The spots are caused by a collapse of the cells of the upper epidermis (Fig. 1). The underlying cells appear normal at first. Later necrosis of the lower epidermis also occurs (Fig. 2). *E*, epidermis; *P*, palisade cells; *M*, mesophyll cells.

Figs. 3 and 4.—Sections through bronze spots on zinc-deficient lucerne leaves similar to those shown in Plate 4, Figure 2. Figure 3 shows numerous small coacervates in cells adjacent to the necrotic upper epidermis, and Figure 4 larger coacervates in the necrotic cells of the upper epidermis. *E*, epidermis; *C*, coacervates.

Figs. 5 and 6.—Sections through lucerne leaves. Figure 5, normal leaf showing well-defined columnar-shaped cells of the palisade layer with large intercellular spaces. Figure 6, zinc-deficient "little" leaf. Disorganization of the palisade layer is evident in the subdivision of the cells and the marked reduction in the intercellular spaces. This probably accounts for the stiff feel of these leaves. *E*, epidermis; *P*, palisade cells; *M*, mesophyll cells; *S*, intercellular space.

PLATE 6

Fig. 1.—First symptom of copper deficiency in lucerne, consisting of a marked epinasty of the petioles of the youngest leaves.

Fig. 2.—Light grey to whitish-coloured necrotic spotting of the younger leaves of copper-deficient lucerne plants. The necrosis usually appears at the base of the leaflet.

Fig. 3.—Death of the top of a copper-deficient lucerne plant following the epinasty of the petioles and white necrosis of the leaflets.

PLATE 7

Portion of the water culture series in which subterranean clover and lucerne were grown together in the same pots. Left to right; complete solution, zinc deficiency, copper deficiency.

THE TEMPERATURE RELATIONS OF *CLOSTRIDIUM BOTULINUM*, TYPES *A* AND *B*

By D. F. Ohye* and W. J. Scott*

[*Manuscript received November* 25, 1952]

Summary

Ten strains of *Cl. botulinum*, type *A*, and 10 of type *B* have been studied at 12 temperatures between 10 and 50°C., and rates of growth measured nephelometrically on sealed cultures. Growth proceeded from spore inocula at temperatures from 15 through to 42.5°C., but not at 12.5 or 45°C. When young, actively growing cultures were transferred to temperatures outside the range permitting spore germination, rates of growth were measured at 12.5, 45, and 47.5°C. After transfer to 10 or 50°C. no sustained growth was observed.

The temperature coefficient of the mean rates of growth was very large at temperatures less than 15°C., gradually decreased to zero at about 40°C., and became negative at higher temperatures. With spore inocula the deduced lag periods were least at 37°C., slightly greater at 42.5, and very much greater at temperatures less than 20°C. The total yield of cells was greatest at about 37°C. and fell considerably as the upper and lower temperature limits were approached. The 20 cultures studied formed a reasonably homogeneous group in their reactions to temperature.

I. Introduction

The extensive, but largely qualitative, observations by early workers led to considerable differences of opinion regarding the most favourable temperatures for growth and toxin production by *Cl. botulinum*. This early work has been reviewed by Meyer (1928) who has also included some of his own results. Meyer has stated that the optimum temperature lies between 25 and 38°C. and is usually about 35°C. The range over which growth may take place is given as 15-55°C.

Since 1928 few additional papers dealing with the effects of temperature on *Cl. botulinum* have been published. Meyer and Gunnison (1929) in a paper on European strains state that growth was equally good at 20 and 37°C., although the lag was greater at the lower temperature. Tanner and Oglesby (1936) studied 21 strains (seven type *A* and 10 type *B*) at 5, 10, 15, and 20°C. Using vegetative inocula they found that all strains grew at 15 and 20°C. At 10°C. there was some growth of five strains although the results were marred by a temporary rise of the incubator temperature to 20°C. No growth occurred at 5°C. When cultures were inoculated with spores, 18 strains grew at 20°C. and only three at 15°C. No development took place at 5 or 10°C. during 10 weeks incubation.

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Tanner and Oglesby concluded that different strains of *Cl. botulinum* "vary materially in their temperature relations" but it is doubtful whether their results actually support such a conclusion. Haines and Elliott (1944) considered the possibility of toxin production at relatively high temperatures. Using six strains (four type *A* and two type *B*) they observed "no appreciable growth" in either of two media over a period of 14 days at 45°C., although six strains of *Cl. sporogenes* all grew within 24 hr. at this temperature. The type of inoculum was, however, not specified. All six strains of *Cl. botulinum* grew well and produced toxin at 37°C.

Although Haines and Elliott studied the time required for detectable amounts of toxin to be liberated at 37°C., they presented no data on the rate of growth of the organism. Mason (1935) quotes a mean generation time of 35 min. as the maximum rate of growth observed for *Cl. botulinum.* This figure was taken from some data on viable counts made by Wagner, Meyer, and Dozier (1925), who had incubated a veal infusion, peptone, glucose broth at 37°C. The data presented by the latter authors are, however, not very suitable for evaluating rates of growth as samples were taken at intervals of 6 hr. and very few of the estimates lie within the logarithmic phase of growth.

It appears that there is a general lack of data on the rates of growth of most species of *Clostridium* and very few figures are available for *Cl. botulinum.* The present paper reports the rates of growth observed when 20 strains of *Cl. botulinum* (10 type *A* and 10 type *B*) were studied at 12 temperatures between 10 and 50°C.

II. METHODS

Cultures were grown in Pyrex test tubes (150 by 15 mm.) sealed under vacuum. The cleaned tubes were first drawn out to a neck, sterilized, and 10 ml. of sterile medium added aseptically. The tubes were sealed immediately after inoculation. Sealing was facilitated by carefully drawing down the neck of the tube to an internal diameter of 1-2 mm. before applying the vacuum. The contents were then boiled under vacuum for a few seconds and the seal completed. The risk of contamination by non-sporing organisms was eliminated by heating the sealed tubes for 10 min. at 80°C. immediately after inoculation.

The light scattered by each tube was then measured in a photoelectric nephelometer and the tube was permanently marked so that it could be replaced in the instrument in the same position. The nephelometer contained a vacuum-type photo-tube collecting light scattered at about 45° from the incident beam, the output of the photo-cell being amplified and measured on a 0-250 D.C. micro-ammeter. The range of the instrument was increased about 80-fold by a selector switch giving five ranges of sensitivity on the micro-ammeter. Readings on blank tubes usually varied between 20 and 50μA. on the most sensitive scale, whereas readings of fully grown cultures were equivalent to some 5000-8000 μA. For readings up to about 5000 μA. the nephelometer reading was directly proportional to the bacterial concentration. Rates of growth (R) were taken as the slopes of the straight lines obtained when logarithms of nephelometer readings (minus the blank values) were plotted against time. Values of *R* are expressed in generations per hour and the lag is evaluated by assuming that *R* is constant (Monod 1949). Calibration curves with eight strains grown at 20, 30, or 37°C. showed that in the linear range each μA. was equivalent to approximately 4.2×10^{-2} μg. of dry cells or $1.75 \pm 0.07 \times 10^5$ cells.

Tubes for incubation were completely immersed in water-baths thermostatically controlled (± 0.05°C.) at the appropriate temperature. For a few of the earlier experiments at intermediate temperatures air thermostats (± 0.5°C.) were used. Baths maintained at temperatures less than 20°C. were operated in cool rooms. This avoided any condensation on tubes which would affect nephelometer readings, and also prevented any possibility of the cultures being temporarily above the temperature of the thermostat.

The medium (N.Y.G.) consisted of 10 g. of neopeptone (Difco), 5 g. of yeast extract (Difco), and 5 g. of glucose dissolved in 1 l. of glass-distilled water and adjusted to pH 7.0. Some experiments were also made with peptic digest (P.D.) medium and cooked meat medium (C.M.M.) (Dubovsky and Meyer 1922). Cultures were inoculated with 10,000 toxin-free viable spores contained in 0.1 ml. phosphate buffer, i.e. 1000 spores per ml. of medium.

Growth outside the temperature range for spore germination was studied in cultures transferred, in the early logarithmic phase, from more favourable temperatures. As the sealed culture was transferred the effects of the temperature change were not complicated by a disturbance of the anaerobic conditions.

Twenty cultures have been studied at all temperatures. Ten of these (3, 33, 38, 62, 109, 134, 172, *L*4, *Q*6, and *Q*7) were type *A* and ten (34, 112, 169, 213, 968, 1787, 3149, *A*12, *L*12, and *T.B.*) were type *B*. The first seven of the type *A* strains and the first four of the type *B* strains were obtained from Prof. K. F. Meyer.

III. RESULTS

(*a*) *Rates of Growth*

Growth occurred from spore inocula at all temperatures between 15 and 42.5°C. although the spores of two (type *B*) strains failed to germinate at 15°C. and six (three type *A* and three type *B*) failed at 42.5°C. Growth of all 20 strains proceeded from spore inocula at all temperatures between 17.5 and 37°C. On the other hand none of the strains grew from spore inocula at 12.5 or 45°C. At 12.5°C. eight (three type *A* and five type *B*) strains grew when spores were germinated and growth was commenced at 20°C., whereas at 45°C. 15 (nine type *A* and six type *B*) strains grew when growth was initiated at lower temperatures. With some of the 15 strains which grew at 45°C. the logarithmic phase was not well defined and steady rates of growth were recorded for only 11 strains (seven type *A* and four type *B*). Five of the strains (three type *A* and two type *B*) which grew at 45°C. also grew at 12.5°C. The mean rates of growth and their standard errors at various temperatures are given separately for each toxigenic type in Table 1. It may be seen that the mean rates are almost identical for the two types at 20 and 25°C. At 30 and 37°C. the mean rates for type *B* strains are slightly greater than for type *A*. At all other temperatures the mean rates are slightly greater for type *A* strains. None of the differences was found to be significant ($P > 0.05$) and, moreover, the variances were similar for each group. The data for both toxigenic types have, therefore, been grouped together.

TABLE 1

MEAN RATES OF GROWTH FOR TYPES *A* AND *B* STRAINS AT VARIOUS TEMPERATURES IN N.Y.G. MEDIUM

Temperature (°C.)	Type *A* Strains			Type *B* Strains		
	No. of Strains	Mean Rate (Divisions/ hr.)	S.E. of Mean	No. of Strains	Mean Rate (Divisions/ hr.)	S.E. of Mean
12·5	3	0·0119	0·0024	5	0·0107	0·0008
15·0	10	0·0359	0·0058	8	0·0346	0·0084
17·5	10	0·131	0·0080	10	0·128	0·0112
20·0	10	0·260	0·0151	10	0·259	0·0208
25·0	10	0·535	0·0166	10	0·533	0·0156
30·0	10	0·917	0·0263	10	0·990	0·0356
37·0	10	1·485	0·0662	10	1·576	0·0492
42·5	7	1·305	0·147	7	1·076	0·137
45·0	7	0·533	0·068	4	0·477	0·074

The average values of *R* for all strains in N.Y.G. medium are shown in Figure 1 for temperatures between 12.5 and 45°C. At the two extremes the rates are, of course, based on experiments with vegetative inocula. At intermediate temperatures the rates are from experiments where spore inocula were used. When all strains grew the value of *R* is the arithmetic mean for 20 strains, but at temperatures where some strains failed to grow the mean is based on the smaller number which grew. The standard error of the mean, expressed as a percentage of the appropriate mean rate, is under 3 per cent. at 30 and 37°C., and rises to between 10 and 12 per cent. at temperatures approaching the upper and lower limits for growth. The variation between the minimum and maximum rates at any one temperature is considerable and amounts to some 30-40 per cent. of the mean at temperatures between 25 and 37°C. As the temperature limits for growth are approached the variation increases and the range exceeds the mean at 15 and 45°C. The mean value of *R* is shown to have a maximum of approximately 1.62 at a temperature close to 40°C. Although no experiments were performed at 40°C. the value of *R* at this temperature is supported by plots of log *R* against the temperature or the reciprocal of the absolute temperature.

Also shown in Figure 1 is the temperature coefficient $1/R.dR/dt$ plotted against the temperature (t). This curve is derived from the values of *R* taken at intervals of 1°C. from the other curve in the figure. It will be noted that the temperature coefficient becomes very large as the minimum temperature is approached. For instance, at 15°C. the rate of growth will be increased by about 50 per cent. per degree rise in temperature, and at 12.5°C. the figure is about 73 per cent. The corresponding values of Q_{10} are approximately 58 and 240. The effects of small changes in temperature are also large as the maximum temperature is approached, the coefficient in this range being negative.

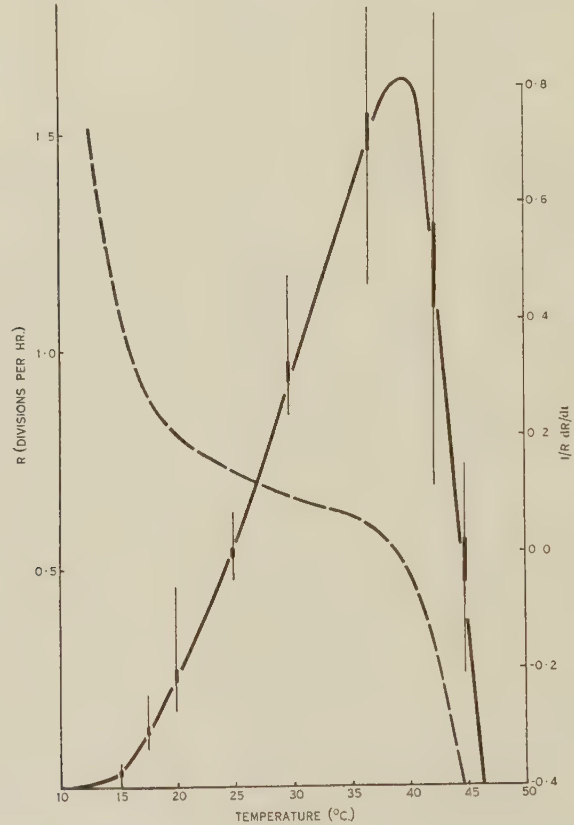

Fig. 1.—Relation between the mean rate of growth (R), the temperature coefficient of R, and the temperature (t) in N.Y.G. medium. (The heavy vertical lines show the mean value of R ± its standard error; the light vertical lines show the range of values observed at each temperature.) ———— Rate of growth. — — — — Temperature coefficient.

(*b*) *Lag*

The duration of the lag period, which includes the time required for germination of the spores, was found to be markedly dependent on temperature. The average results for all experiments are shown in Figure 2 in which the logarithm of the lag is plotted against temperature. The average lag was found to be about 5 hr. at 37°C., over a day at 20°C., and almost a week at 15°C. The value at 42.5°C. was significantly greater than at 37°C. At all temperatures there was close agreement between duplicate tubes and fair agreement between strains, but a larger variation between experiments carried out on different occasions. It may be concluded, therefore, that uncontrolled variations between different batches of medium had greater effect on the lag than variations in the condition of the inoculum, or in the degree of anaerobiosis effected during sealing of the tubes.

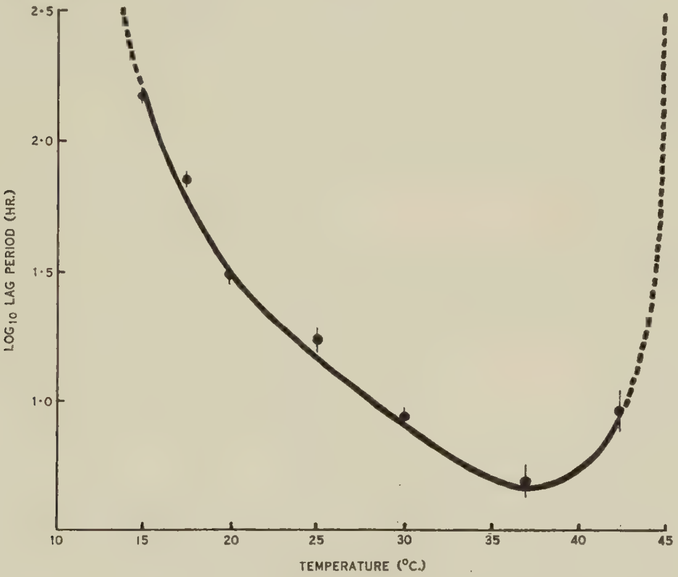

Fig. 2.—Relation between the lag period and temperature (N.Y.G. medium). The vertical lines indicate the mean ± its standard error.

(*c*) *Yield of Cells*

Data on the maximum size of crop were obtained from nephelometer readings taken in the early stationary phase. Readings during the late stationary phase were often as much as 70 per cent. less than the maximum readings. This phenomenon, which is presumably due to lysis, is much more pronounced, and has a more rapid onset, at the higher temperatures. Cells from cultures showing this fall in turbidity have frequently undergone partial disintegration, they stain only faintly, and show no evidence of incipient sporulation. The average results for all experiments are given in Figure 3. Yields of less than about 200 μg. dry weight per ml. are within the linear range of the measuring instrument, and will be somewhat more reliable than for the denser cultures where the dry weight-nephelometer relationship is no longer linear. The data are nevertheless adequate to demonstrate a marked effect of temperature on the total yield of cells.

(*d*) *Lower Temperature Limits*

In the present experiments almost all strains developed from a spore inoculum at 15°C. At this temperature 10 (five type *A* and five type *B*) of the cultures which grew were tested for the presence of toxin after incubation for 40 days. Titres of the order of 10^3-10^4 mouse-doses per ml. were observed with all the type *A* cultures. All but one of the type *B* cultures proved toxic to mice, the titre being of the order of 10-100 mouse-doses per ml. At 10 and 12.5°C. evidence was obtained that the spores did not germinate. After incubation at 12.5°C. for 3 months and after 6 months at 10°C. the sealed tubes were immersed in a water-bath at 80°C. for 10 min. Subsequently the tubes were transferred to 30°C. Growth occurred in all tubes after a normal lag. The original inoculum had, therefore, remained resistant to 80°C. and the medium was still suitable for the growth of *Cl. botulinum.*

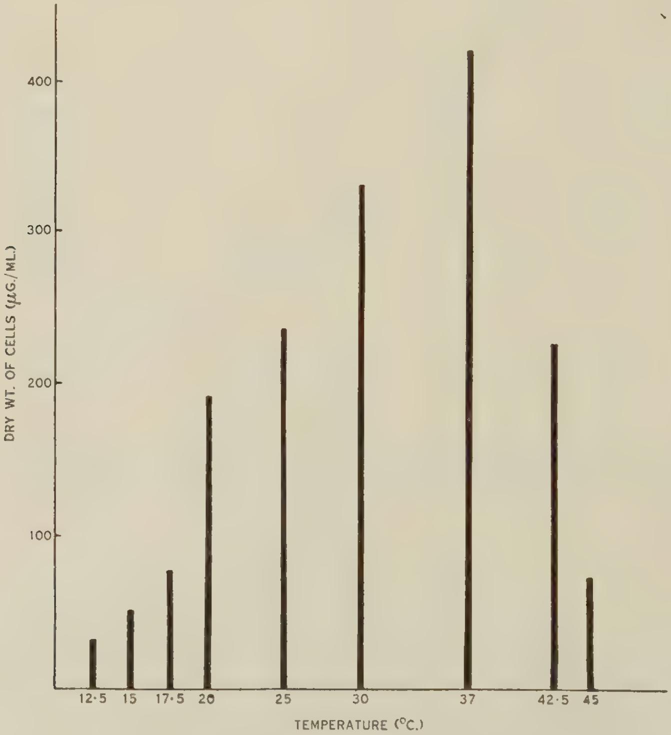

Fig. 3.—Relation between the yield of cells and temperature (N.Y.G. medium).

When growth was commenced at 20°C. and the tubes subsequently transferred, during the early logarithmic phase, to 12.5°C. growth of some strains continued at the lower temperature. As the tubes were completely immersed in the water-bath they would be cooled to within 0.1°C. of the lower temperature within 5 min. of the transfer. It was, nevertheless, often observed that during an initial period of some 2-8 hr. the rate of growth was much greater than that maintained thereafter. During this initial period the rate of growth was sometimes almost as great as at 20°C., but the extent of growth was never greater than one generation and more usually only one-quarter to one-half of this amount. Subsequently, a much slower steady rate of growth was maintained over several days and it is this lower rate shown in Figure 1. The higher initial rate is presumably at the expense of metabolites or cellular components already accumulated during the early growth at 20°C. An indication of some of the different responses of cultures transferred to 12.5°C. is given in Figure 4. There is evidently need for caution in deciding which rate is appropriate to the steady state at 12.5°C., but it is suggested that the lower rates are the best values available at present.

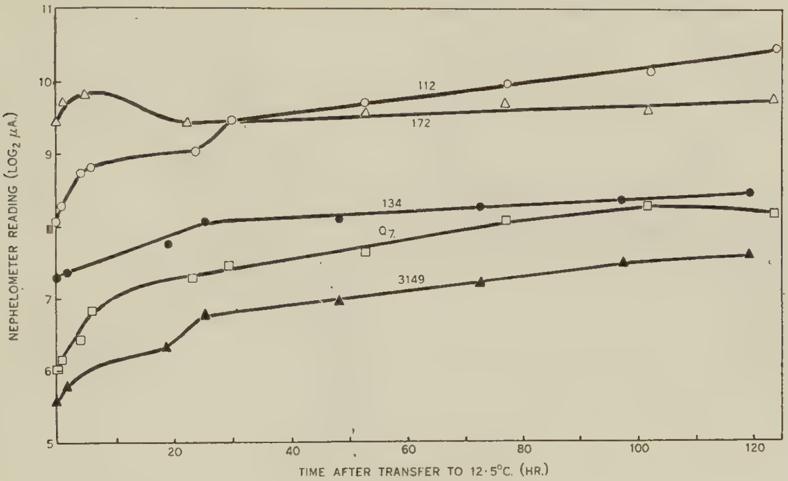

Fig. 4.—Growth of various strains at 12.5°C. after transfer from 20°C. (N.Y.G. medium).

When cultures were transferred from 20 to 10°C. there was little or no increase in the first few hours and subsequently very little change in turbidity during a period of 10 days. There was, however, a general tendency for a very slow increase during the first 5 or 6 days at 10°C. followed by constant or slowly declining readings of turbidity. Growth was too slow for rates to be measured reliably and the amount was limited to approximately 1.5 generations during the period of observation. It is believed that this small increase reflects the utilization of metabolites previously synthesized at 20°C. rather than a real capacity to proliferate at 10°C.

(*e*) *Upper Temperature Limits*

There was no growth from spore inocula in N.Y.G. medium held at 45°C. for 50 days, nor in P.D. medium held for 40 days. With both media the inoculum survived 10 min. at 80°C. after incubation, and growth occurred when the tubes were transferred to 37°C. The evidence is, therefore, that the spores had failed to germinate and that the media remained suitable for growth. Many of these cultures were transferred back to 45°C. after growth had commenced at 37°C. Growth then ensued at 45°C. even though the spores had previously failed to germinate in these same tubes at the same temperature. When cultures in which growth had commenced at 30 or 37°C. were transferred to 45°C. the results were rather variable. In many cultures a high initial rate approximately equal to the rate at 37°C. was maintained for 1-2 hr. This was followed by a gradual or sometimes rather abrupt change to a lower constant rate. In other cultures the initial high rate was not detected or was evident to only a slight extent. Some growth curves illustrating the different types of response are shown in Figure 5. Both the media used showed similar types of response, but the rates of growth were somewhat higher on P.D. medium.

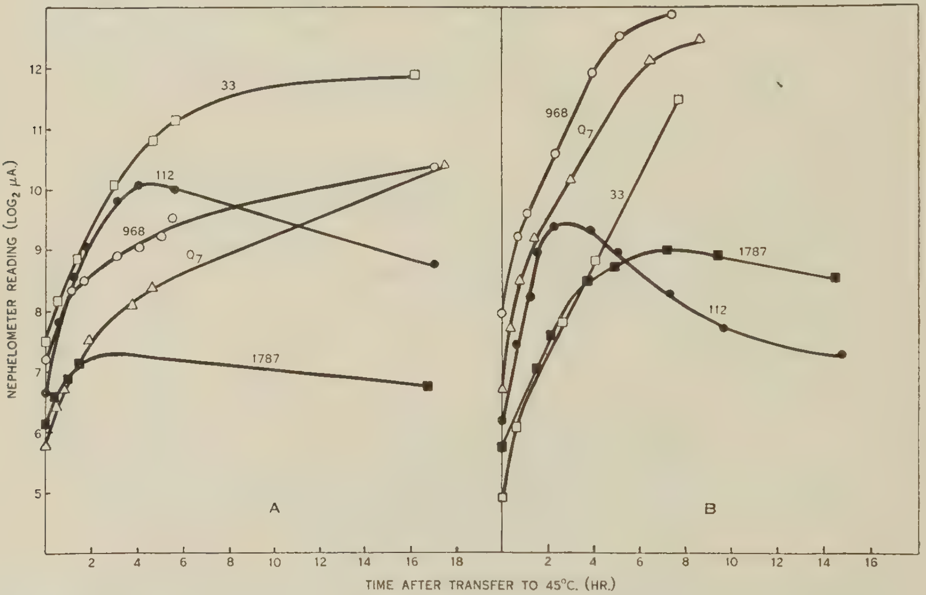

Fig. 5.—Growth of various strains at 45°C. after transfer from 37°C. *A*, N.Y.G. medium; *B*, P.D. medium.

Similar experiments have been performed by transferring cultures from 30 or 37°C. to 47.5°C. Some typical results are shown in Figure 6. At this temperature there is no continued growth on the N.Y.G. medium apart from the initial high rate maintained for 1-2 hr. On the P.D. medium most strains show no sustained growth beyond the first 2 hr. but some strains do continue to grow at rates varying between 0.2 and 0.3 approximately. At 47.5°C. and, to a lesser extent, at 45°C., gas production is much less marked than at lower temperatures.

When cultures were transferred to 50°C. there was no growth apart from a very small rise in turbidity during the first 30 min. Thereafter the readings remained constant or steadily fell.

Two additional experiments were performed in C.M.M. In the first of these nine strains (two type *A* and seven type *B*) were inoculated with the usual spore inoculum and after sealing under vacuum were incubated at 37°C. for 6 hr. This period was selected as sufficient to allow germination of spores and the beginning of vegetative growth. The cultures were then transferred to 50°C. for 7 days. After opening, 0.2 ml. aliquots were injected into mice and 0.5 ml. aliquots into duplicate tubes of pork infusion thioglycollate agar.

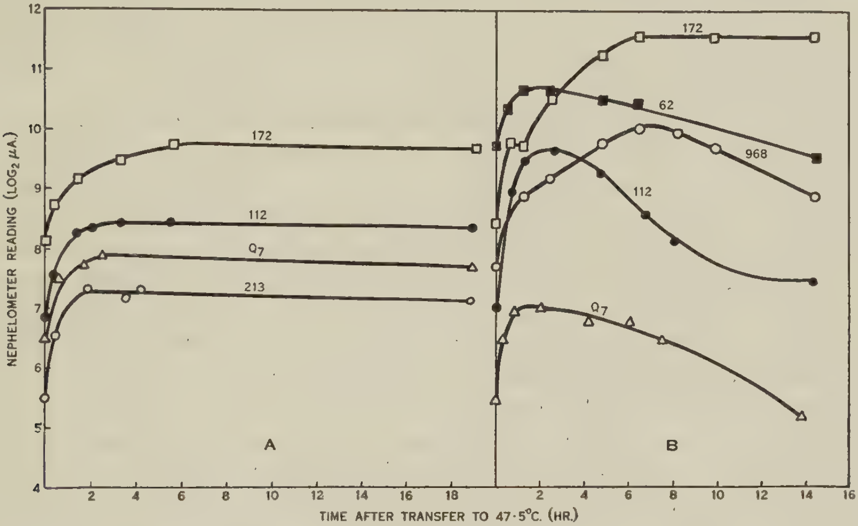

Fig. 6.—Growth of various strains at 47.5°C. after transfer from 37°C. *A,* N.Y.G. medium; *B,* P.D. medium.

All the mice remained well and free from symptoms of botulism. No viable organisms were detected in two cultures and in others the count, after 3 days at 37°C., varied from 2 to 45 per ml. The cultures showed no loss of vacuum during incubation at 50°C., no signs of digestion of the meat, nor was there any microscopic evidence that growth had occurred. The pH varied between 6.9 and 7.0. In the second experiment seven of the above nine strains (two *A* and five *B*) were inoculated into C.M.M., using 0.5 ml. ($> 10^8$ cells) of a C.M.M. culture grown for 20 hr. at 37°C. The cultures were held for 7 days at 50°C. Again there was no macroscopic or microscopic evidence of growth, and no toxin was detected. The pH was between 6.8 and 6.9. Viable counts were not done, but all cultures were returned to 37°C. for 5 days. Growth ensued in one culture only, the original inoculum in the remaining cultures having died during the 7 days at 50°C.

The data show that growth is adversely affected by a temperature of 45°C. and still more so at 47.5°C. although some strains in one medium still were capable of limited growth at the higher temperature. No evidence of growth was obtained at 50°C. and, indeed, it appears that vegetative inocula die at this temperature.

IV. Discussion

The present results confirm the report of Tanner and Oglesby (1936) that vegetative growth may proceed at temperatures somewhat lower than those permitting germination of spores. They also show that a similar situation exists in the upper temperature range, vegetative growth being possible at temperatures above the limits for spore germination. Trustworthy as these facts might appear it should, nevertheless, be recognized that very little is known about the basic conditions which determine the chances of successful germination of spores. For this reason it would be wise to place some reservations on the observations that spore germination did not occur at 12.5 or 45°C. Under different environmental conditions germination might occur at these temperatures.

When the range of temperatures over which vegetative growth occurs is considered, the present results are in good agreement with earlier workers regarding the lower limits, but there is some disagreement about the maximum temperatures. Although Tanner and Oglesby (1936) report some growth at 10°C. there was some uncertainty in their control of temperature which may have affected their results. The present experiments show a very large temperature coefficient which is increased as the temperature is lowered from 15 to 12.5°C. Even if, as is most unlikely, the temperature coefficient showed no further increase at lower temperatures the predicted rate of growth at 10°C. would correspond to one division in about 400 hr. This very low rate would be even lower if the temperature coefficient increased in the manner to be expected. It seems, therefore, safe to conclude that the probability of growth at 10°C. is very small. At the upper end of the temperature range the position is much less certain. Greer (1924) has reported growth of one strain each of type *A* and type *B* at 50 and 55°C. in beef heart medium under a vaseline seal. Apart from gas production the methods by which growth was assessed were not recorded, and no information is given on the accuracy with which temperatures were controlled. Meyer (1928), in a brief reference to his own observations, affirms that incubation at 55°C. produces involution forms and weak toxin but the experimental conditions under which these results were obtained are not given. The present experiments do not support the view that growth occurs at 55°C. and, indeed, have not yielded evidence of growth at 50°C. It is difficult to account for the discrepancy.

In the experiments now reported there can be little doubt about the reliability of the temperature control. Furthermore, there is clear evidence that in the media used growth at 45°C. was more irregular and slower than at 37°C. At 47.5°C. growth was even more restricted and, on one medium, was not continued beyond a period of about 2 hr. after transfer to this temperature. The trends in the temperature coefficient shown in Figure 1 substantiate the view that sustained growth on N.Y.G. medium is extremely unlikely at 50°C.

The evidence that growth at 45 and 47.5°C. is rather better on P.D. medium deserves some comment. There is no information which would show whether the improved performance on this medium is due to more efficient synthetic processes, or to a reduced rate of destruction of essential enzymes or metabolites. For this reason one is unable to reach any worth-while conclusions regarding the probabilities of growth at still higher temperatures under different environmental conditions. The possibility of growth at somewhat higher temperatures is, however, worthy of serious consideration in the light of the effects of the ionic environment and of increased hydrostatic pressure on the thermostability of some enzymes.

All the strains examined showed their greatest rates of growth around 37-40°C. and the greatest yield of cells at similar temperatures. There is, therefore, no support for the view of many of the early workers that some strains had optimum temperatures less than 30°C. Many of these reports were based on estimates of the toxicity and it is most probable that the results were really a measure of other factors which are now known to affect the stability of the toxins. The strains used in these experiments have been isolated from widely different localities over a period of many years and may, therefore, be considered as representative. A point of some importance is that no large differences in the temperature requirements of the strains were detected, and no consistent differences between the type *A* and *B* strains could be demonstrated. Some differences in the temperature relations of the different strains do undoubtedly exist, but it is believed that these are relatively minor and that the emphasis should more properly be placed on the relative homogeneity of the group in their reactions to temperature. For this reason one would not expect the reactions of other type *A* and *B* strains to differ substantially from those described in this paper. It should, however, not be concluded that other toxigenic types of *Cl. botulinum* would also show similar behaviour. In this respect it is pertinent to draw attention to the observation by Dolman *et al.* (1950) that a type *E* strain grew and produced toxin at 6°C. This finding has been confirmed with all type *E* strains tested and the results will be published in a later paper.

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STUDIES ON *AEROBACILLUS POLYMYXA*

IV. FACTORS AFFECTING THE PRODUCTION OF HYDROGENASE AND THE HYDROGENLYASE SYSTEMS

By W. G. CREWTHER*

[*Manuscript received September* 23, 1952]

Summary

Once-washed cells of *Aerobacillus polymyxa* harvested from a medium containing peptone, yeast extract, glucose, phosphate, and magnesium sulphate, did not produce hydrogen from formate, glucose, or pyruvate nor was methylene blue reduced in the presence of gaseous hydrogen. Production of hydrogen from formate could be demonstrated in fermenting whole wheat mash and by once-washed cells of *A. polymyxa* grown on strained 10 per cent. wheat mash.

The glucose, peptone, yeast extract medium was found to yield active washed-cell preparations when ferrous sulphate was included in the medium, maximum activity of hydrogenase and of formic, glucose, and pyruvic hydrogenlyases being obtained with approximately 0.05, 0.20, 0.05, and 0.15 per cent. ferrous sulphate respectively. Relatively high concentrations of yeast extract and potassium phosphate were also required for optimum activity.

Media containing suspensions of inert material such as kaolin, calcium carbonate, or paper pulp produced active washed-cell suspensions having different degrees of activity.

Low concentrations of ferrous salts in the medium have been shown to increase the activity of the hydrogenlyase systems in fermenting cultures of *A. polymyxa*. It is suggested, however, that at high concentrations of ferrous salts the insoluble sludge, formed by ferrous ions with phosphate and components of the yeast extract, protects the cells during washing, either from oxidation or from complete leaching out of some essential component of the enzyme systems.

I. INTRODUCTION

Stickland (1929), Stephenson and Stickland (1932), and Yudkin (1932) have demonstrated the production of hydrogen from formate, glucose, and glycerol by "resting cell" suspensions of *Escherichia coli* and other bacterial species, and Koepsell and Johnson (1942) and Davies (1942) found pyruvate to be a substrate for the production of hydrogen by cells of *Clostridium acetobutylicum*. Investigations by Donker (1926), and by Adams and Stanier (1945), have shown that *A. polymyxa* produces hydrogen and carbon dioxide during the fermentation of media containing glucose or various other mono- or disaccharides.

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In this laboratory, initial attempts to demonstrate production of hydrogen from formate, glucose, or pyruvate by washed-cell suspensions of *A. polymyxa* harvested from a medium containing glucose, peptone, yeast extract, and salts, were unsuccessful, although the production of hydrogen during growth of the cells in such a medium has been demonstrated. The present paper describes methods for the preparation of washed suspensions of *A. polymyxa* showing hydrogenlyase activity.

II. Methods

Cells of *A. polymyxa* (strain GO(7), Crewther 1950) were harvested by centrifuging 500 ml. of the culture after 20 hr. incubation at 30°C. The cells were washed once in 20 ml. of 0.1M phosphate buffer at pH 6.5, centrifuged, and suspended in 20 ml. of the same buffer solution. Hydrogen evolution was estimated by placing 2-ml. aliquots of cell suspension in the main compartment of Warburg flasks containing 0.5 ml. of substrate solution (1.0M sodium formate, 0.1M glucose, or 0.1M sodium pyruvate at pH 6.5) in the side bulb, and a filter paper roll with 0.4 ml. of 20 per cent. KOH in the centre compartment. The atmosphere was replaced with oxygen-free nitrogen. After equilibration at 30°C. and tipping the flasks, evolution of hydrogen was followed by the usual procedure. Hydrogenase activity was estimated by a similar procedure using an atmosphere of O_2-free H_2 and 0.5 ml. of 1 per cent. methylene blue in the side bulb as hydrogen acceptor. On tipping the flasks the uptake of molecular hydrogen was measured. Dehydrogenase activities were estimated by the Thunberg technique using methylene blue at a final concentration of 0.02 per cent. Iron was assayed by a modification of the method of Kennedy (1927).

III. Experimental

In order to determine whether actively fermenting cells of *A. polymyxa* produce hydrogen from formate, 2-ml. aliquots of a well-mixed culture of the organism in 10 per cent. strained wheat mash containing 1 per cent. calcium carbonate were placed in Warburg flasks and the atmosphere replaced with nitrogen. The side bulbs contained 0.5 ml. water or 1M sodium formate, and KOH papers were in the centre cups. The production of hydrogen with time was followed, and, after an initial lag period during which no hydrogen appeared, the rate of hydrogen production in each flask increased to a constant value. The formate or water was then tipped into the culture. The rate of hydrogen production immediately increased to a new constant value in flasks containing formate whereas addition of water had no effect (Fig. 1).

In view of the report by Waring and Werkman (1944) that an adequate supply of iron salts is essential for the production of formic dehydrogenase, hydrogenase, and formic hydrogenlyase by *E. coli*, the effects of adding ferrous sulphate to glucose peptone medium were studied, using the following basal medium:

Glucose	10 g.
Difco peptone	10 g.
Difco yeast extract	5 g.
KH_2PO_4	2 g.
K_2HPO_4	2 g.
$MgSO_4.7H_2O$	0.25 g.
Tap water	1000 ml.

$FeSO_4.7H_2O$ was added in amounts up to 3.0 per cent. and cells grown on these media were collected and washed by the standard procedure. Hydrogen evolution by these preparations from formate, glucose, and pyruvate was found to be a function of the amount of ferrous sulphate added to the medium. Figure 2 shows that optimal rates of hydrogen production, using formate, glucose, and pyruvate as substrates, were obtained with media containing 0.20, 0.05, and 0.15 per cent. of added $FeSO_4.7H_2O$ respectively. These optimum concentrations were found to vary somewhat in different experiments and with different strains of *A. polymyxa* but were of this order. Viable cell counts and direct

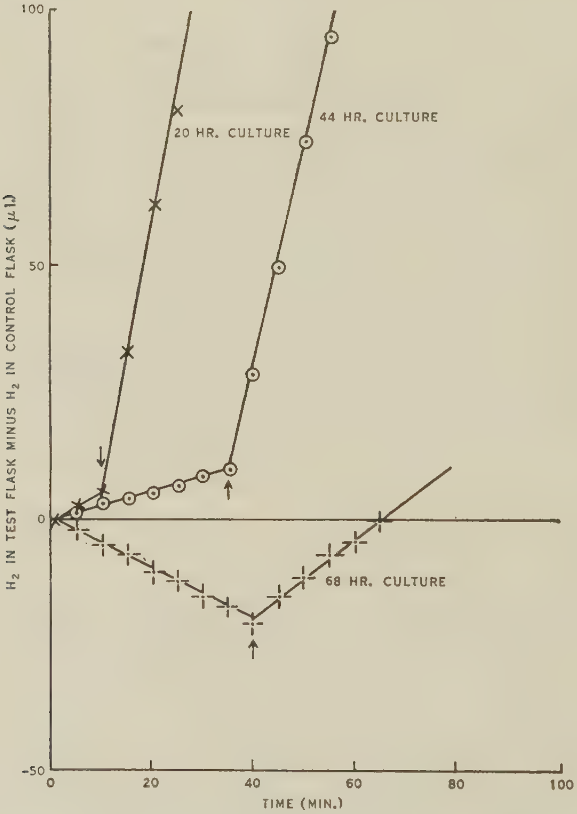

Fig. 1.—Increase in the rate of production of hydrogen on addition of formate to a fermenting wheat mash culture of *A. polymyxa*. Arrows indicate the time of addition of formate to the cultures.

microscopic counts of the cultures and suspensions of *A. polymyxa* showed that changes in cell density were not responsible for the results obtained (Table 1).

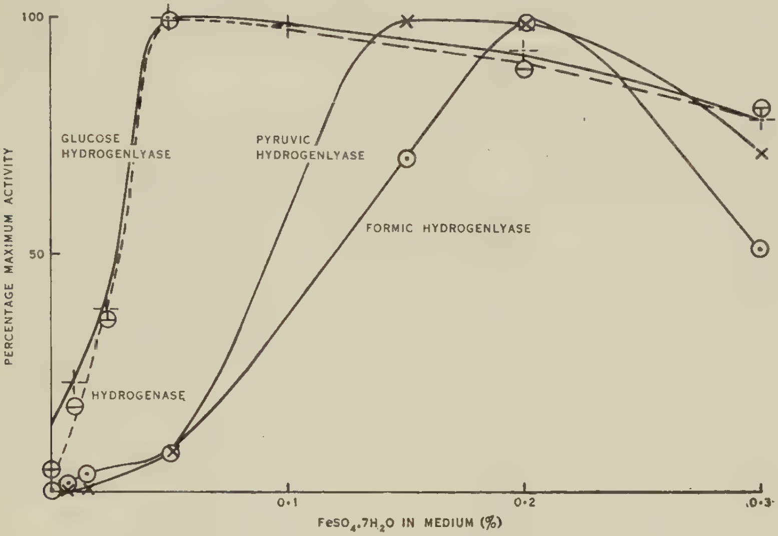

Fig. 2.—Effect of ferrous sulphate in the growth medium on the activity of formic hydrogenlyase and related enzymes of washed-cell suspensions of *A. polymyxa.* Maximum activities against formate, glucose, pyruvate, or hydrogen are in the ratio 100:55:20:70.

In a similar manner it was found that the hydrogenase activity of once-washed cell suspensions varied with the ferrous sulphate content of the medium, optimum activity being obtained at about 0.05 per cent. $FeSO_4.7H_2O$ (Fig. 2). In some experiments it was possible to detect hydrogenase and glucose hydrogenlyase activity in washed cells grown in the absence of added ferrous sulphate.

TABLE 1

SOLUBLE IRON CONTENT OF MEDIA BEFORE AND AFTER GROWTH OF *A. POLYMYXA*

Total Fe Added (mg./100 ml.)	Viable Cell Count of Culture ($\times 10^{-7}$)	Soluble Fe Before Growth (μg./100 ml.)	Soluble Fe After Growth (μg./100 ml.)	H_2 Evolved from Formate in 30 Min. (cu. mm.)
nil	60	22	110	0
0·6	9	46	202	2
2·0	5	111	544	4
6·0	5	146	652	7
20·1	4	183	568	17
60·3	5	107	800	135

Estimations by the Thunberg technique of the dehydrogenase activity of cells grown on media containing varying amounts of ferrous salts were found to be misleading, owing to an appreciable slowing of the reduction of methylene blue, which varied in extent with the amount of precipitate. However, washed-cell suspensions of *A. polymyxa* grown on media containing no ferrous sulphate were found to possess an active "glucose dehydrogenase," whereas formic and pyruvic dehydrogenases were not sufficiently active to be detected with certainty by the Thunberg method.

The sludge of insoluble iron salts consisted partly of ferrous hydroxide, partly of basic ferrous phosphate together with an insoluble iron compound of a component of yeast extract. The soluble iron content of the media before and after growth of *A. polymyxa* was determined, the original medium and a 20-hr. culture being filtered through No. 42 Whatman papers for this purpose. The results of these estimations (Table 1) show a considerable increase in soluble iron concentration after growth of bacteria. This is probably due largely to a decrease in pH and possibly in part to the formation of soluble organic iron compounds by the microorganism. The formation of coordination complexes with fermentation products may also be partly responsible for the increase in soluble iron. Iron salts could not be successfully replaced by salts of other related divalent metals at concentrations that did not inhibit growth. The presence of this sludge, which contained nitrogenous compounds, made it impossible to interpret the results obtained in terms of unit mass of bacteria.

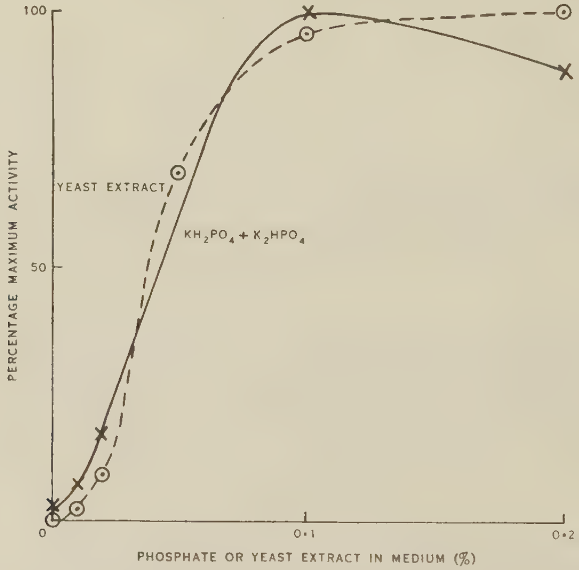

Fig. 3.—Effect of potassium phosphate and yeast extract in the growth medium on the activity of the formic hydrogenlyase of washed cell suspensions of *A. polymyxa*. A mixture of equal proportions of KH_2PO_4 and K_2HPO_4 was used and the values in the figure refer to the final concentration of each of these salts in the medium.

Since both phosphate and yeast extract contributed to the precipitate formed in the medium, experiments were conducted to determine whether they were required for production of hydrogenlyase activity and whether by reducing their concentration higher concentrations of soluble iron could be made available to the organism. The basal medium containing 0.1 per cent. $FeSO_4.7H_2O$ was used for these experiments, the yeast extract concentration being held constant and phosphate varied or vice versa. From Figure 3, it is evident that both yeast extract and phosphate are required for production of active cell suspensions, the optimum concentrations of each being of the order of that used in the original basal medium.

TABLE 2

PROTECTION OF THE FORMIC "HYDROGENLYASE" SYSTEM OF *A. POLYMYXA* BY INSOLUBLE MATERIAL IN THE GROWTH MEDIUM

A. polymyxa Grown on Basal Medium Containing:	H_2 Evolved in 30 Min. (cu. mm.)
No addition	0
$FeSO_4.7H_2O$ 0·2%	51
$KAl(SO_4)_2H_2O$ 0·1% ..	0
$CaCO_3$ 1·0%	14
Kaolin 1·0%	55
Pulped filter paper 1·0% ..	5

Attempts were made to replace the yeast extract of the basal medium with up to 2 μg./ml. nicotinic acid, nicotinamide, riboflavin, folic acid, pyridoxine hydrochloride, thiamine hydrochloride, sodium pantothenate, pyridoxamine, pyridoxal, and biotin, both singly and collectively. In some experiments pyridoxal and pyridoxamine increased the activity of the washed suspensions to some extent, but in no case did the suspensions approach the activity of cells grown in the presence of yeast extract. Glutamate, glutamine, and asparagine were also without effect in the absence of yeast extract. It seems likely that the action of yeast extract is chiefly one of increasing the cell count in the final culture rather than a specific increase in the hydrogenlyase activity per unit mass of bacteria, the addition of yeast extract to a glucose-peptone medium containing 0.2 per cent. $FeSO_4.7H_2O$ causing increases in the viable cell count of up to 100-fold. Smaller increases in count were observed in media containing pyridoxamine and pyridoxal.

In view of the fact that strained wheat mash and the ferrous sulphate medium which produced active cell suspensions of *A. polymyxa* both contained some form of sediment, six flasks were prepared containing the basal medium with the addition of 0.01 per cent. $FeSO_4.7H_2O$ (normally insufficient to give a washed-cell suspension producing hydrogen from formate), to five of which additions of 1 per cent. $CaCO_3$, 1 per cent. acid-washed kaolin, 1 per cent. pulped filter paper, 0.1 per cent. $KAl(SO_4)_2.H_2O$, or 0.2 per cent.

$FeSO_4.7H_2O$ were made. After inoculation, incubation, and collection by the usual procedure it was found that active washed suspensions were obtained from the flasks containing calcium carbonate or kaolin, particularly the latter. Some slight activity was also obtained in the preparation containing filter paper (Table 2). The bacteria were largely enveloped in a mixture of the sediment and slime, which necessarily reduced the effectiveness of washing.

In order to evaluate the actual increase in the activity of the enzyme system responsible for the evolution of hydrogen from formate due to the incorporation of $FeSO_4.7H_2O$ in the growth medium, the rate of production of hydrogen from 2-ml. aliquots of fermenting basal medium containing varying amounts of $FeSO_4.7H_2O$ was determined before and after addition of formate.

As in the initial experiments of this type, the rate of production of hydrogen was followed until it became constant, the substrate was then added and the increased constant rate of hydrogen evolution measured. Figure 4 indicates that cultures containing 0.003 per cent. $FeSO_4.7H_2O$ possessed maximum formic hydrogenlyase activity. Concentrations of the order of 0.2 per cent. $FeSO_4.7H_2O$ inhibited the production of hydrogen.

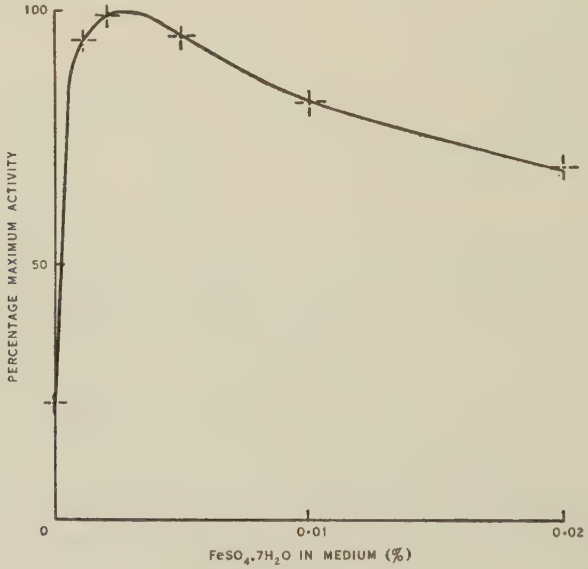

Fig. 4.—Effect of ferrous sulphate in the medium on the rate of production of hydrogen by a fermenting culture of *A. polymyxa* on addition of formate. Sodium formate solution was added to 2-ml. aliquots of the culture after a constant rate of hydrogen production had been reached and the final constant rate of evolution was measured.

IV. Discussion

The results shown in Figure 4 indicate that addition of small amounts of ferrous sulphate to the basal medium increases the activity of the hydrogenlyase and hydrogenase systems in a fermenting culture of *A. polymyxa*, whereas higher concentrations actually decrease the production of these enzymes or inhibit their activity. The role of high concentrations of ferrous salts in producing active washed-cell suspensions appears to be the formation of a sludge of insoluble iron salts which protects the enzyme system during the process of collection and washing of the bacteria. The production of a copious polysaccharide slime by *A. polymyxa* undoubtedly assists such a protective mechanism and makes difficult the thorough washing and dispersion of the bacteria. This view is substantiated by the production of active washed-cell suspensions from media containing kaolin or calcium carbonate.

These results appear to be related to those of Yudkin (1932) who found that, although a particular strain of *E. coli* produced hydrogen during the fermentation of a glucose medium with an inorganic nitrogen source, washed-cell suspensions from this medium failed to produce hydrogen from formate or glucose. Yudkin attributed this to the washing out of some stabilizing substance in the medium.

It is also noteworthy that whereas formic and pyruvic hydrogenlyases required the addition of approximately 0.2 per cent. ferrous sulphate to the medium to ensure optimal activity of washed suspensions, 0.05 per cent. $FeSO_4.7H_2O$ was sufficient to produce a protective sludge with glucose hydrogenlyase and hydrogenase. Glucose dehydrogenase did not require protection during washing whereas formic and pyruvic dehydrogenase activities were absent from washed cells obtained from media containing no added iron salts. All three dehydrogenases have been demonstrated in washed-cell suspensions from media containing kaolin, and it seems probable therefore that the dehydrogenases form an integral part of the hydrogenlyase systems. The evidence for this view is not sufficient to disprove Stephenson and Stickland's suggestion that the hydrogenlyases are distinct from the dehydrogenases and hydrogenase (1932), but suggests a reinvestigation of the problem.

V. Acknowledgments

The author wishes to thank Mr. J. B. Bingley of the Division of Animal Health and Production, C.S.I.R.O., for carrying out analyses of the soluble iron content of the culture media.

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STUDIES ON *AEROBACILLUS POLYMYXA*

V. THE SIGNIFICANCE OF THE MICHAELIS CONSTANTS OF THE HYDROGENLYASE SYSTEMS AND RELATED ENZYMES

By W. G. Crewther[*]

[*Manuscript received September* 23, 1952]

Summary

Washed-cell suspensions of *Aerobacillus polymyxa* have been used to determine the Michaelis constants of the enzyme systems producing molecular hydrogen from formate, glucose, and pyruvate and of the dehydrogenases acting on these substrates. For each substrate the K_m value of the dehydrogenase was considerably smaller than for the analogous hydrogenlyase.

A theoretical examination of the significance of the K_m values of a model dehydrogenase system showed that different values might be expected according to the concentration and nature of the hydrogen acceptor used.

It was shown experimentally that substitution of rosinduline, potassium indigo trisulphonate, or phenol indophenol for methylene blue in the Thunberg technique resulted in a wide range of K_m values for the formic dehydrogenase of *Escherichia coli*. There was also a significant variation in the results obtained with different concentrations of methylene blue.

It has been concluded that discrepancies in the K_m values of dehydrogenases and the corresponding hydrogenlyases of bacteria cannot be used as evidence of the existence of hydrogenlyases as enzymes distinct from the dehydrogenases and hydrogenase of bacteria.

I. Introduction

Stephenson and Stickland (1932) have postulated the existence in certain bacteria of enzymes, the hydrogenlyases, which catalyse the production of molecular hydrogen from substrates such as formate and glucose. Their experimental evidence supported the hypothesis that the hydrogenlyases are distinct from the corresponding dehydrogenases and hydrogenase. The experimental data adduced by these authors included the observation that the Michaelis constants of the formic dehydrogenase and of the formic hydrogenlyase of *Escherichia coli* were of a different order of magnitude, the former being considerably smaller than the latter. Ordal and Halvorson (1939) suggest that, as the Michaelis constant evaluates the dissociation constant of the enzyme-substrate complex, this observation supports the hypothesis of a separate hydrogenlyase system though they consider their own results to favour the opposite view.

In a previous paper of this series (Crewther 1953) it has been shown that, under suitable conditions, washed suspensions of *Aerobacillus polymyxa*

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produce hydrogen from formate, glucose, and pyruvate. The results obtained suggested that hydrogen may arise from formate and glucose as a result of the consecutive action of the specific dehydrogenase and hydrogenase, though no conclusive evidence was presented.

In developing the expression

$$r = \frac{[S]\ .\ [E]\ .\ k}{[S] + K_m}$$

$$= \frac{[S]\ .\ R}{[S] + K_m} \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

for the rate of an enzyme catalysed reaction (r being the rate of the reaction, [S] and [E] the concentrations of substrate and enzyme respectively, R the maximum reaction rate, K_m the dissociation constant of the enzyme substrate complex, and k another constant), Michaelis and Menten (1913) assumed that an equilibrium existed between substrate, free enzyme, and the enzyme-substrate complex. On the assumption that a steady state is set up between enzyme, substrate, and enzyme-substrate complex, Briggs and Haldane (1925) have developed a similar equation for the rate of an enzymic reaction of the form

$$r = \frac{[S]\ .\ R}{[S] + \frac{k'_1 + k_2}{k_1}}, \qquad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

in which k_1, k'_1, and k_2 are the rate constants for the reactions

$$\begin{array}{lll} S + E \longrightarrow ES & & (k_1) \\ ES \longrightarrow E + S & & (k'_1) \\ ES \longrightarrow E + A + B & & (k_2). \end{array}$$

Consequently the value obtained by determining the concentration of substrate giving half the maximum overall reaction rate will be $(k'_1 + k_2)/k_1$ and the error incurred in assuming that $K_m = k'_1/k_1$ is greatest when the rate of decomposition of the complex is great relative to its rate of dissociation into enzyme and substrate.

Application of the theory of reaction rate kinetics to a system consisting of a specific dehydrogenase catalysing the transfer of hydrogen or electrons to a carrier, the reduced form of which serves as substrate for the enzyme, hydrogenase, provides an expression in the form of a quadratic in "r", the steady state reaction rate. This is due to the fact that the reduced carrier, which is a product of the first reaction, is the substrate of the second; consequently the reactions of this intermediate cannot be neglected in the way Michaelis and Menten neglect the effects of the products of the reaction. The value of the substrate concentration giving half maximum activity would have little meaning in such a system. This therefore constitutes a theoretical reason for questioning the validity of a comparison of the K_m values of the hydrogenlyase systems and dehydrogenases of bacteria.

In applying the theory to the Thunberg technique the following simple reactions are postulated:

$$\begin{aligned} \mathrm{E} + \mathrm{SH_2} &\rightleftharpoons \mathrm{ESH_2} \\ \mathrm{ESH_2} + \mathrm{D} &\rightleftharpoons \mathrm{ES} + \mathrm{DH_2} \\ \mathrm{ES} &\rightleftharpoons \mathrm{E} + \mathrm{S}, \end{aligned}$$

and on attainment of a steady state

$$\begin{aligned} r &= k_1\,[\mathrm{E}]\,[\mathrm{SH_2}] - k'_1\,[\mathrm{ESH_2}] \\ r &= k_2\,[\mathrm{ESH_2}]\,[\mathrm{D}] - k'_2\,[\mathrm{ES}]\,[\mathrm{DH_2}] \\ r &= k_3\,[\mathrm{ES}] - k'_3\,[\mathrm{E}]\,[\mathrm{S}] \\ [\mathrm{E_T}] &= [\mathrm{E}] + [\mathrm{ESH_2}] + [\mathrm{ES}]. \end{aligned}$$

Eliminating [ESH$_2$], [ES], and [E], the expression for r becomes

$$\frac{k'_1k_3[\mathrm{E_T}](k_1k_2k_3[\mathrm{D}][\mathrm{SH_2}]-k'_1k'_2k'_3[\mathrm{S}][\mathrm{DH_2}])}{(k_2k_3[\mathrm{D}]+k'_1k_3+k'_1k'_2[\mathrm{DH_2}])(k'_1k_3+k_1k_3[\mathrm{SH_2}]+k'_1k'_3[\mathrm{S}])-(k_3-k'_1)(k_1k_2k_3[\mathrm{D}][\mathrm{SH_2}]-k'_1k'_2k'_3[\mathrm{S}][\mathrm{DH_2}])}.$$

If $k'_2[\mathrm{DH_2}]$ and $k'_3[\mathrm{S}]$ are comparatively insignificant,

$$r = \frac{k_1k_2k_3[\mathrm{E_T}][\mathrm{D}][\mathrm{SH_2}]}{[\mathrm{SH_2}]\;(k_1k_3 + k_1k_2[\mathrm{D}]) + k'_1k_3 + k_2k_3[\mathrm{D}]}$$

and when $r = \tfrac{1}{2}\,.\,\dfrac{k_2k_3[\mathrm{E_T}][\mathrm{D}]}{k_3 + k_2[\mathrm{D}]}$, $[\mathrm{SH_2}] = \dfrac{k'_1k_3 + k_2k_3[\mathrm{D}]}{k_1k_3 + k_1k_2[\mathrm{D}]}$,

it follows that K_m would vary with [D]. As [D] increased, K_m would approach k_3/k_1 and as [D] approached zero K_m would approach k'_1/k_1, the dissociation constant of the enzyme-substrate complex. At high concentrations of the hydrogen acceptor, D, K_m would be practically independent of its concentration and of its chemical structure. However, the concentration at which this desirable condition is attained would depend on the relative magnitudes of k_2 and k'_1 and of k_2 and k_3, i.e. it would depend on the chemical nature of the hydrogen acceptor. At low concentrations of D both its concentration and chemical nature — E_0 value, pK values, and steric properties — would be expected to affect the value of K_m obtained.

This paper therefore compares the K_m values of the hydrogenlyase and dehydrogenase systems of *A. polymyxa* and *E. coli*, and demonstrates the influence of the concentration and nature of the hydrogen acceptor used in the Thunberg determination of dehydrogenase activity on the values of K_m obtained for these enzyme systems.

II. EXPERIMENTAL

Washed-cell suspensions of *A. polymyxa* strain GO(7)) were prepared and tested as previously described (Crewther 1953). Media for growing *A. polymyxa* contained 0.2 per cent. $\mathrm{FeSO_4.7H_2O}$ unless otherwise stated. Cells of *E. coli* (strain 557 R) grown on 500 ml. basal medium containing no yeast extract (Crewther 1953) were washed and suspended in 200 ml. buffer.

In determining the effect of substrate concentration on the rate of hydrogen production it was observed that the rate of evolution was practically constant during the period of the experiments. Consequently it was unnecessary to plot hydrogen production against time to obtain the initial reaction rate. Curves relating formate concentration to the rate of hydrogen production are shown in Figure 1, and it will be observed that significant differences occur from one experiment to another. The chief difference appears to lie in the concentration of formate at which inhibition occurs. Similar curves for the production of hydrogen from glucose and pyruvate, and for the corresponding dehydrogenases, are plotted in Figures 2 and 3. The curves for pyruvic hydrogenlyase and dehydrogenase are seen to be atypical and resemble that obtained for pyruvic hydrogenlyase of *Clostridium acetobutylicum* by Davies (1942). It was found that in some cases normal curves were obtained (Fig. 2) when the cells of *A. polymyxa* were grown on a medium in which $FeSO_4$ was replaced by 1 per

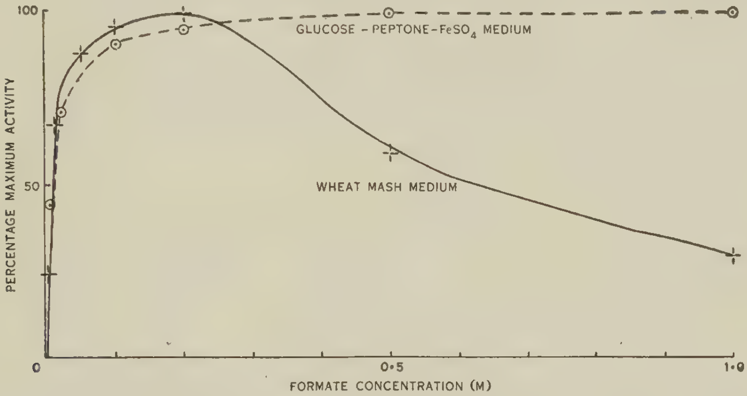

Fig. 1.—Relationship between substrate concentration and activity of the formic hydrogenlyase system of *A. polymyxa* grown on wheat mash and on glucose-peptone-ferrous sulphate medium.

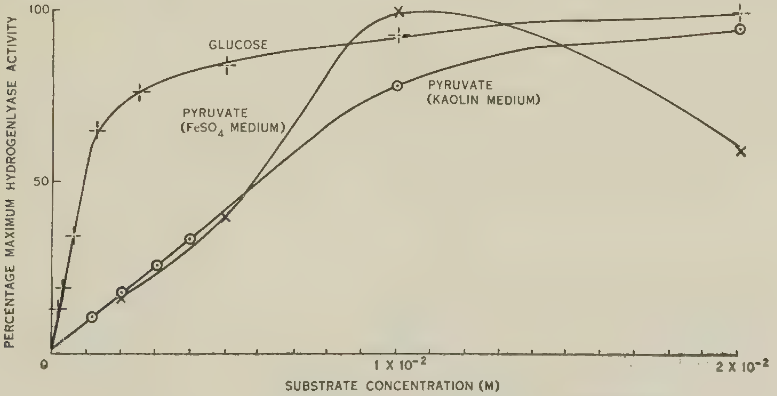

Fig. 2.—Relationship between substrate concentration and activity of glucose hydrogenlyase and pyruvic hydrogenlyase of *A. polymyxa*. Experiments with pyruvic hydrogenlyase were done on cells grown on glucose peptone medium containing ferrous sulphate and on similar medium containing 1 per cent. kaolin but only 0.005 per cent. $FeSO_4.7H_2O$.

cent. kaolin (Crewther 1953). The values of K_m obtained from the atypical curves of Figures 2 and 3 will have doubtful significance but are included for comparison.

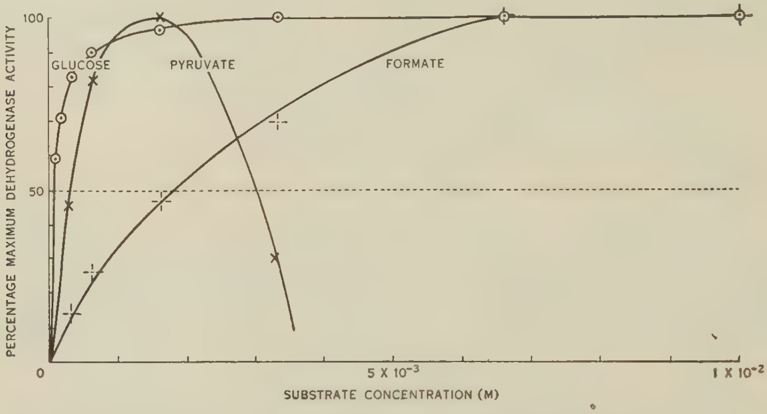

Fig. 3.—Relationship between substrate concentration and the activity of formic, glucose, and pyruvic dehydrogenase systems of *A. polymyxa*. A final concentration of 0.02 per cent. methylene blue was used in the dehydrogenase estimations.

The K_m values obtained in this way are summarized in Table 1, the constants for the dehydrogenases being considerably smaller in each case than those of the corresponding hydrogenlyase. Stickland's observation (1929) that the K_m value of the formic dehydrogenase of *E. coli* is less than that of the hydrogenlyase has therefore been confirmed with *A. polymyxa*.

Table 1

K_m VALUES OF THE DEHYDROGENASES AND HYDROGENLYASES OF *A. POLYMYXA*

Substrate	Michaelis Constant (M)	
	Dehydrogenase	Hydrogenlyase
Formate	$1\cdot8\times10^{-3}$	$1\cdot0\times10^{-2}$
Glucose	$6\cdot0\times10^{-5}$	$9\cdot0\times10^{-4}$
Pyruvate	$3\cdot0\times10^{-4}$	$6\cdot0\times10^{-3}$

The theoretical investigation of the significance of the K_m values obtained by the Thunberg technique indicated that the value obtained would depend on the concentration of the dyestuff used as hydrogen acceptor, and on its chemical structure. In order to test this experimentally, *E. coli* was used because of the greater stability of the hydrogenlyase systems of this organism, and to eliminate the need for a protecting sludge in the cell suspensions. In each case reduction of nine-tenths of the original amount of dye present was used as an end-point. The curves relating substrate concentration and the rate of reduction of methylene blue, using 0.02, 0.002, and 0.001 per cent. methylene blue in the final mixtures are shown in Figure 4. The K_m values obtained from these curves were 1.9×10^{-3}, 1.1×10^{-3}, and 1.9×10^{-4}M respectively. In a similar manner, considerable differences were obtained in the curves when using final concentrations of 0.5×10^{-5}M rosinduline, potassium indigo trisulphonate, methylene blue, and phenol indophenol, with E'_0 values at pH 7.0 of -0.254, -0.083, $+0.011$, and $+0.227$ V. respectively (Fig. 5). The K_m values obtained in this way for formic dehydrogenase of *E. coli* varied from 8×10^{-5} to 5×10^{-3}M.

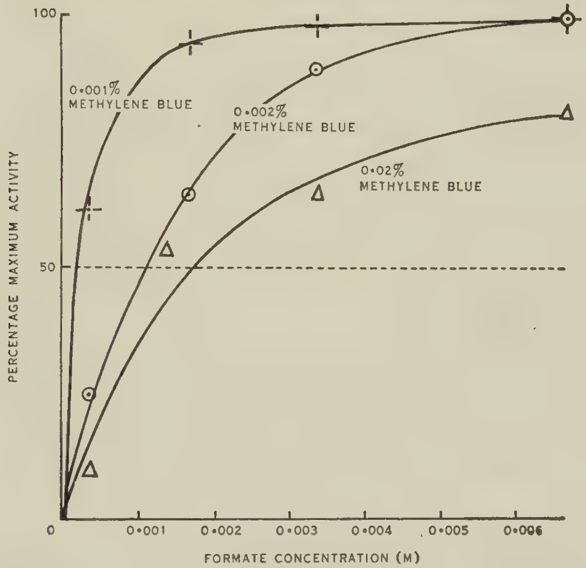

Fig. 4.—The use of different concentrations of methylene blue as hydrogen acceptor in the determination of the K_m value of the formic dehydrogenase system of *E. coli.* The final concentrations of methylene blue are indicated.

III. Discussion

As predicted by a theoretical examination of the reactions taking place in the estimation of dehydrogenase activity by the Thunberg technique, the experimental data presented in this paper show clearly that the K_m values obtained for the formic dehydrogenase system of *E. coli* are dependent on the nature and the concentration of the hydrogen acceptor used in estimating dehydrogenase activity. The theoretical examination of the significance of the K_m values of the hydrogenlyase systems of bacteria has shown that, if hydrogen is produced as the result of the consecutive action of a specific dehydrogenase linked to hydrogenase by a suitable carrier, the equation evaluating the rate of the reaction as a function of substrate concentration would not have the form of the classical Michaelis-Menten equation. Consequently the experimental values for the Michaelis constant would not bear a simple relationship to the dissociation constant of the enzyme-substrate complex. For these reasons the differences in K_m value of the hydrogenlyase systems and the corresponding dehydrogenase systems do not conflict with the view that gaseous hydrogen arises as the result of the consecutive action of a dehydrogenase and the enzyme hydrogenase.

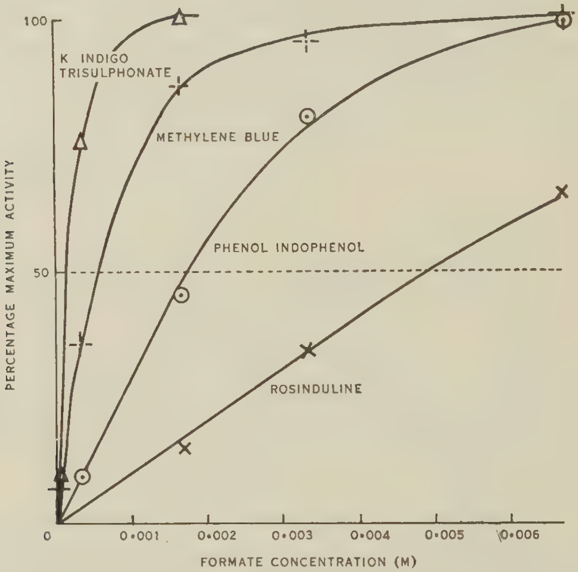

Fig. 5.—The use of different hydrogen acceptors in determining the K_m value of the formic dehydrogenase system of *E. coli.*

The existence of the hydrogenlyases as separate enzymes has been questioned by Tasman and Pot (1935), Ordal and Halvorson (1939), and Waring and Werkman (1944), but the experimental facts on which the concept of the hydrogenlyases was originally based (Stephenson and Stickland 1932) have not been satisfactorily explained by another hypothesis. The present paper and the preceding paper of this series (Crewther 1953) suggest that further investigation of the problem is necessary.

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STUDIES ON *AEROBACILLUS POLYMYXA*

VI. SOME PROPERTIES OF THE HYDROGENLYASE SYSTEMS OF BACTERIA

By W. G. Crewther*

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Summary

The formic hydrogenlyase, glucose hydrogenlyase, and hydrogenase systems of *Aerobacillus polymyxa* were inactivated by dilution, repeated washing, or oxygenation of a washed-cell suspension of the organism. The hydrogenase and glucose hydrogenlyase systems were less sensitive than the formic hydrogenlyase, and during inactivation by oxygenation the relationship between the activities of these enzymes was rectilinear over the lower range of enzyme activities. Formic dehydrogenase and pyruvic dehydrogenase were both readily inactivated by the above procedures whereas the enzyme system transferring hydrogen from glucose to methylene blue was unaffected.

Suspensions of *Escherichia coli* were relatively stable, though prolonged oxygenation caused gradual inactivation of the formic dehydrogenase and formic hydrogenlyase systems, the relationship between these activities being rectilinear over almost the entire range of activities. Hydrogenase and glucose hydrogenlyase of *E. coli* were practically unaffected by oxygenation.

The difference in susceptibility to oxygenation of the enzymes of *A. polymyxa* and *E. coli* is attributed to the fact that in the presence of glucose the former absorbs oxygen at a much greater rate, relative to the rate of transfer of hydrogen from glucose to an acceptor, than does the latter organism.

Inactivation of the formic hydrogenlyase system of *A. polymyxa* by washing was prevented or lessened by glucose, ethanol, glycerol, and sulphite, the latter particularly in the presence of a suitable redox indicator. It is suggested that these substances prevent oxidation of essential components in the labile enzyme system by maintaining the effective redox potential at a low level. Glutamate, which maintains a low redox potential in the cell suspension, was ineffective as the result of a secondary inhibition, found to be characteristic of all dicarboxylic acids. Oxidizing agents, reagents complexing or reacting with heavy metals, and substances reacting with sulphydryl groups were also inhibitory. The rate of production of hydrogen from mixtures of glucose and formate at concentrations greater than 0.2M was less than the rate of hydrogen evolution from the substrates separately.

The effects of oxidation have been explained in terms of hydrogen production by a system consisting of a dehydrogenase and hydrogenase linked by suitable carriers. Formic dehydrogenase, being more labile to oxidation than hydrogenase, chiefly determines the overall formic hydrogenlyase activity, whereas the glucose dehydrogenase system is very stable to oxidation and the activity of the glucose hydrogenlyase system is therefore determined mainly by the activity of the more labile hydrogenase.

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I. Introduction

In a previous paper (Crewther 1953*a*) it has been reported that washed-cell suspensions of *Aerobacillus polymyxa* grown on glucose, peptone, yeast extract medium do not produce hydrogen from formate unless a sludge of kaolin, insoluble ferrous compounds, or other finely divided material is present in the bacterial suspension. It was suggested that the sludge prevented inactivation of the cells either by limiting oxygenation of the suspension or by hindering the removal of some stabilizing substance present in the culture medium.

The present study has been carried out to investigate the stability of the formic hydrogenlyase system of *A. polymyxa* and the effects of various inhibitors on it. These data have been compared with similar data for related enzyme systems in the bacteria.

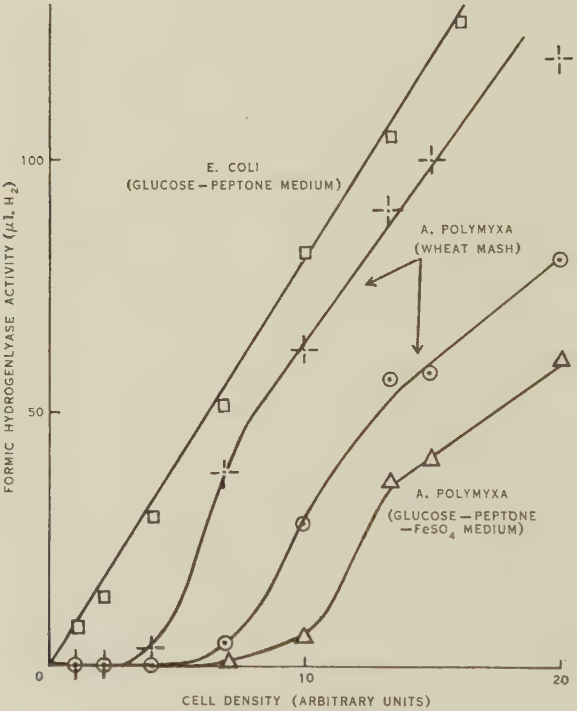

Fig. 1.—Effect on the formic hydrogenlyase system of diluting washed-cell suspensions of *A. polymyxa* and *E. coli.*

II. Methods

The methods of growing, collecting, and washing the cells of *A. polymyxa* (strain GO(7)) and *Escherichia coli* (strain 557 R) and the methods of estimating enzyme activities were those described previously (Crewther 1953*a*, 1953*b*). Unless otherwise stated the cells were washed once by the standard procedure.

III. EXPERIMENTAL AND RESULTS

(*a*) *Effects of Diluting and Washing the Cell Suspension*

To determine whether the hydrogenlyase systems of *A. polymyxa* contain an essential diffusible component, a washed-cell suspension was diluted to produce a series of cell suspensions of differing cell density and these were used for the determination of formic hydrogenlyase activity. In Figure 1 the rates of hydrogen evolution of these suspensions have been plotted against the cell density, using an arbitrary unit for the latter. Curves obtained using different suspensions of *A. polymyxa* are seen to vary but are characterized by a rapid decrease in activity at a certain dilution. No such divergence from linearity occurred with the particular strain of *E. coli* used in these experiments (Fig. 1).

Repeated washing of the bacterial suspension with phosphate buffer at pH 6.5 also caused almost complete inactivation of the formic hydrogenlyase of *A. polymyxa* (Table 1), yet no appreciable reactivation occurred when portion of the wash liquor, or wash liquor from a very heavy bacterial suspension, was returned to the cell suspension. It is apparent also from Table 1 that the time during which the cells remain suspended in the wash liquor before centrifuging is important in determining their final activity. When ice-cold buffer was used for washing and suspending the cells a greater proportion of the original activity was retained.

TABLE 1

INACTIVATION OF THE FORMIC HYDROGENLYASE OF *A. POLYMYXA* BY WASHING

Cells washed in 0.1M phosphate buffer at pH 6.5, a 2-ml. aliquot taken, the suspension centrifuged, and the supernatant replaced by an equal volume of buffer

No. of Washings	Hydrogenlyase Activity (% of once-washed cells)	
	Cells in Buffer 10 Min. before Centrifuging	Cells Centrifuged Immediately
1	100	100
2	Nil	15
3	Nil	7
4	Nil	Nil

Dilution experiments with the hydrogenase and glucose hydrogenlyase (Fig. 2) of *A. polymyxa* and *E. coli* provided curves similar to the dilution curves for formic hydrogenlyase, the enzyme system of *E. coli* again providing a rectilinear relationship between activity and cell density. Repeated washing of *A. polymyxa* decreased the hydrogenase activity, but to a lesser extent than the formic hydrogenlyase activity.

(*b*) *Effects of Oxygenation*

The relative stability of the enzyme systems of *A. polymyxa* and *E. coli* to oxygenation is indicated in Table 2. Once-washed cells of each organism were subjected to agitation with O_2 and H_2 at room temperature and the activity of the resulting suspensions compared with a suspension which had not

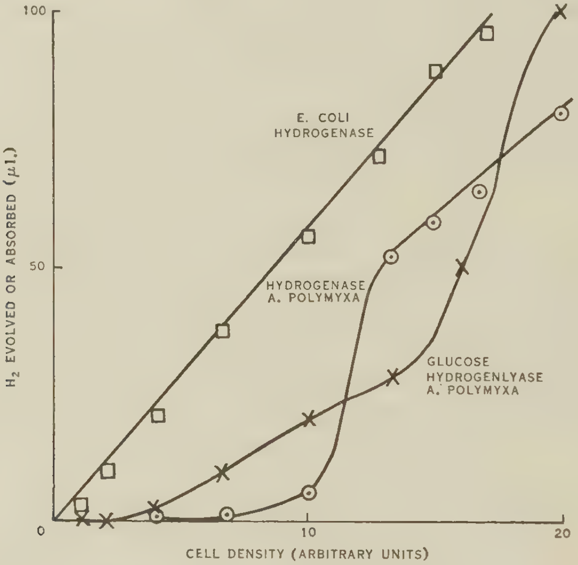

Fig. 2.—Effect on the hydrogenase and glucose hydrogenlyase systems of diluting washed-cell suspensions of *A. polymyxa.*

Table 2

INACTIVATION OF THE FORMIC AND GLUCOSE HYDROGENLYASE SYSTEMS AND HYDROGENASE OF *A. POLYMYXA* AND *E. COLI* BY OXYGENATION

Species	Gasification of Bacterial Suspension	Hydrogenase Activity (% of untreated)	Formic Hydrogenlyase Activity (% of untreated)	Glucose Hydrogenlyase Activity (% of untreated)
*A. polymyxa**	Nil	100	100	100
	H_2, 15 min.	102	113	103
	O_2, 5 min.	101	43	100
	O_2, 10 min.	65	6	87
	O_2, 15 min.	31	Nil	60
E. coli	Nil	100	100	100
	H_2, 30 min.	102	101	100
	O_2, 30 min.	100	58	102

* The volumes of hydrogen evolved or assimilated by 2 ml. of untreated bacterial suspension in the presence of formate, glucose, and methylene blue were of the order of 100, 55, and 70 μl. in 30 min. respectively.

been agitated in this way. In general there was a slight increase in activity of hydrogenase and formic and glucose hydrogenlyase when agitated with hydrogen. Oxygen caused a rapid inactivation of the formic hydrogenlyase of *A. polymyxa* and a more gradual inactivation of the hydrogenase and glucose hydrogenlyase systems; the corresponding enzymes of *E. coli* were relatively stable to oxygen, hydrogenase, and glucose hydrogenlyase, being virtually unaffected by 30 min. oxygenation, and the formic hydrogenlyase system losing 40 per cent. of its original activity by this treatment.

TABLE 3

EFFECT OF HYDROGEN AND OXYGEN ON THE ACTIVITY OF THE FORMIC, GLUCOSE, AND PYRUVIC DEHYDROGENASE OF *A. POLYMYXA*

Treatment of Cells	Substrate	Time of Decolourization (min.)
H_2 through suspension 30 min.	Water	>90
	Formate	10
	Glucose	12
	Pyruvate	16
O_2 through suspension 30 min.	Water	>90
	Formate	>90
	Glucose	12
	Pyruvate	45

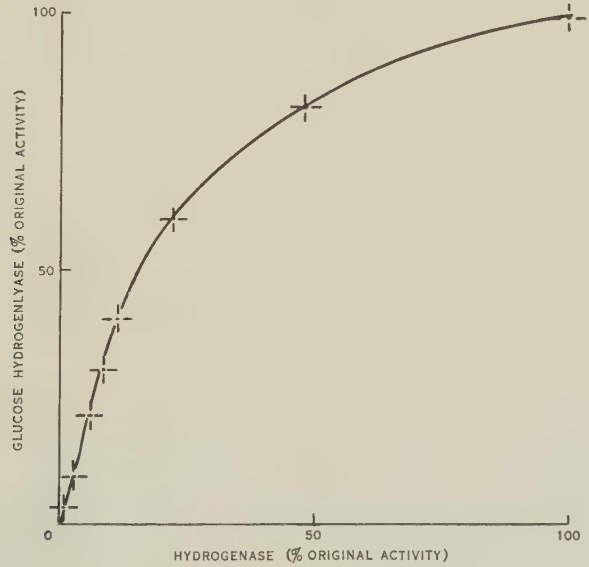

Fig. 3.—Relationship between hydrogenase activity and glucose hydrogenlyase activity during aeration of a suspension of *A. polymyxa*.

The formic and pyruvic dehydrogenases of *A. polymyxa* were also sensitive to oxygen. A comparison of the rates of reduction of methylene blue by oxygenated and un-oxygenated cell suspensions of *A. polymyxa* in the presence of formate, glucose, and pyruvate demonstrated the almost complete inactivation of the formic dehydrogenase and partial inactivation of the pyruvic dehydrogenase. On the other hand the enzyme system transferring hydrogen from glucose to methylene blue was practically unaffected by oxygenation (Table 3).

In order to determine whether a quantitative relationship exists between the residual activity of hydrogenase and the glucose hydrogenlyase of *A. polymyxa* after oxygenation, a cell suspension was assayed for both enzyme systems at intervals during oxygenation, and it was found that as inactivation of the cells progressed the relationship between the activity of the two enzyme systems approached a straight line (Fig. 3) and was in fact rectilinear, within experimental error, over the lower range of activities.

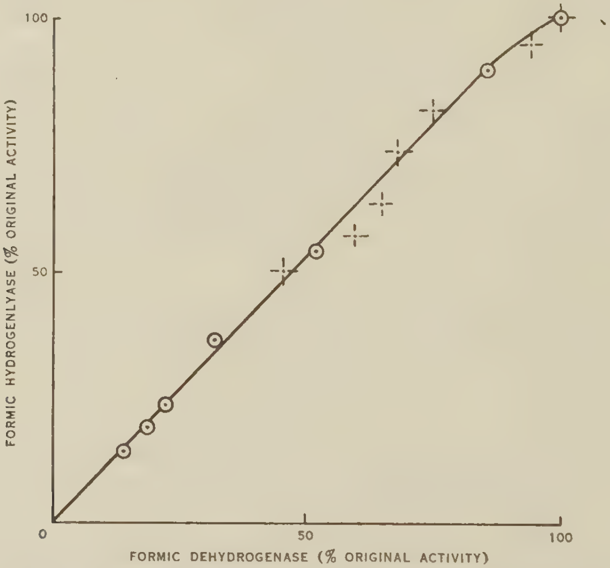

Fig. 4.—Relationship between the activity of the formic hydrogenlyase system and the dehydrogenase system of *E. coli* during aeration.

A similar experiment with *E. coli* was not feasible owing to the relative stability of both systems involved. A comparable experiment with the formic hydrogenlyase of *A. polymyxa* would be very inaccurate owing to rapid changes in activity during estimation. A comparison of the formic hydrogenlyase activity and the formic dehydrogenase activity of *E. coli* during oxygenation provided the curve of Figure 4. In this instance the relationship is rectilinear within experimental error over the major proportion of the activity. As a basis of comparison the reduction in activity of the formic dehydrogenase, the hydrogenase, and the glucose hydrogenlyase activity of *E. coli* are shown in Table 4. The dehydrogenase activity was reduced to less than 15 per cent. of the original without significant alteration in the hydrogenase activity or glucose hydrogenlyase activity.

(*c*) *Substances Protecting the Hydrogenlyase System*

The above data suggest that inactivation of formic hydrogenlyase takes place by oxidation, and that this is assisted by removal of certain components of the cells or surrounding fluid. This was confirmed by the observation that the supernatant liquid after removal of the cells from a culture of *A. polymyxa* effectively stabilized the formic hydrogenlyase system (Table 5). The stabilizing effect of culture fluid and other preparations was determined by rapidly washing cells of *A. polymyxa* three times in cold buffer, adding the substance

Table 4

INACTIVATION OF THE FORMIC DEHYDROGENASE AND HYDROGENASE OF *E. COLI* BY OXYGENATION

Period of Oxygenation (min.)	Formic Dehydrogenase Activity (% of original)	Hydrogenase Activity (% of original)	Glucose Hydrogenlyase Activity (% of original)
nil	100	100	100
5	88	100	101
10	78	99	101
20	60	100	99
40	26	99	101
60	11	99	100
80	10	98	99

Table 5

STABILIZATION OF HYDROGENLYASE OF *A. POLYMYXA* BY CONSTITUENTS OF THE CULTURE MEDIUM

Flasks contained 1 ml. thrice-washed suspension of cells, 1 ml. stabilizing agent, 0.5 ml. 1M formate

Stabilizing Agent	H_2 Evolved (cu. mm.)
Water	10
Yeast extract	10
Straub's flavoprotein	9
Rat liver extract	106
Boiled rat liver extract	47
Crude coenzyme I	8
Crude coenzyme II	9
First wash liquor	12
Culture medium (after removing cells)	175
Boiled culture medium	160
Medium evaporated to half volume	152
Ethanol extract of medium (ethanol removed under reduced pressure)	130
Ethanol 0·1M	56
Ethanol 0·001M	53

to be tested, and incubating for 20 min. in the Warburg flasks at 30°C. before replacing the air with nitrogen. Table 5 shows yeast extract, Straub's flavoprotein, crude preparations of coenzymes 1 and 2, and the first wash liquor, to be without effect. The culture medium, after removal of cells, effectively stabilized the formic hydrogenlyase system, and its effect was only slightly decreased by boiling or evaporation to remove volatile components. The ethanol-soluble components of the medium were also effective, and ethanol itself had a considerable stabilizing effect (Table 5). These results, and Davies' (1942) use of glucose to stabilize the fermentation systems of *Clostridium acetobutylicum* suggested the use of glucose in this instance. Table 6 shows that a final concentration of 0.005M glucose was almost as effective as a concentrated culture supernatant in stabilizing formic hydrogenlyase and that it had a similar effect on hydrogenase. The fact that less hydrogen was absorbed in the presence of methylene blue with cells stabilized in the presence of 0.05M glucose than with cells in 0.005M glucose can be explained in terms of the tendency for hydrogen to be evolved from the glucose. However, no such explanation can be found for the smaller amount of hydrogen evolved from formate in the presence of 0.5M glucose than in the presence of 0.005M glucose.

Table 6

STABILIZATION OF THE FORMIC HYDROGENLYASE AND HYDROGENASE OF THRICE-WASHED CELLS OF *A. POLYMYXA* BY GLUCOSE

Flasks contained 1.0 ml. cell suspension, 1.0 ml. solution of stabilizing agent, 0.5 ml. 1M formate

Stabilizing Agent	*Hydrogenase Activity (cu. mm. H_2 absorbed)	*Formic Hydrogenlyase Activity (cu. mm. H_2 evolved)
Water	0	9
Glucose 1M	13	36
Glucose 0·01M	18	73
Glucose 1M (no formate)	—	21 (glucose hydrogenlyase)
Concentrated culture medium ..	—	86

* Experiments with hydrogenase and hydrogenlyase conducted with different cell suspensions.

Repetition of the dilution experiments using phosphate buffer, and phosphate buffer containing 0.001 and 0.01M glucose for washing and suspending the cells of *A. polymyxa* by the standard technique, provided the curves of Figure 5. The divergence from a rectilinear relationship between formic hydrogenlyase activity and cell density is seen to decrease with increasing glucose concentration, little deviation being apparent in the presence of 0.01M glucose. However, though the dilution effect can be attributed largely to dilution of glucose remaining in the cells after washing, it was found that repeated washing of a cell suspension with 0.01M glucose resulted in an appreciable progressive inactivation (Fig. 6) which was frequently preceded by an increase in activity after washing one to three times.

Glycerol, pyruvate, and sulphite have also proved effective stabilizing agents. Sulphite, which was found to be inhibitory to an active cell suspension, stabilized a thrice-washed suspension to some extent, but was much more effective in the presence of a suitable dyestuff such as haematein. Table 7 indicates almost complete stabilization of the formic hydrogenlyase system in the presence of 0.005 per cent. haematein and 0.02M sodium sulphite (taking account of the inhibition of the enzyme by sulphite of this concentration (Table 10)).

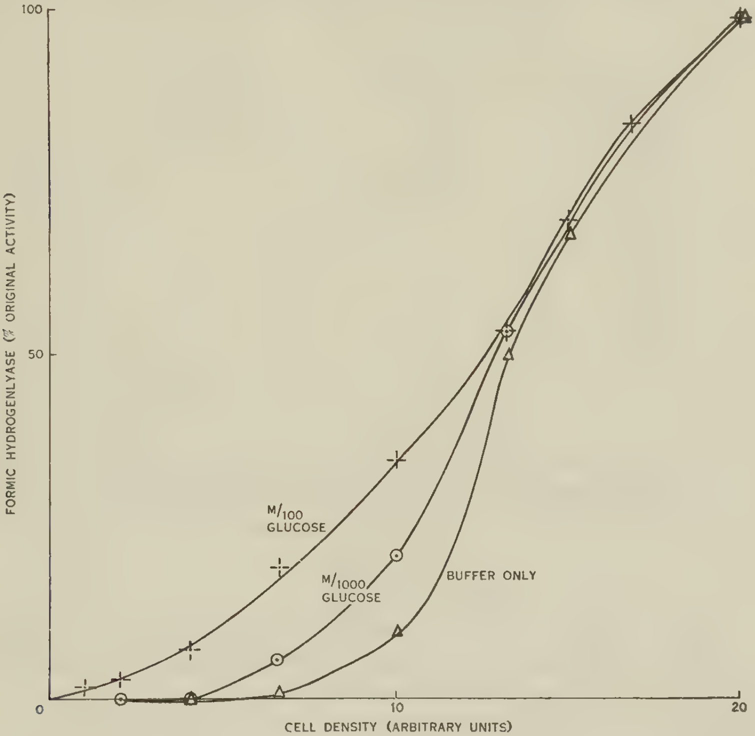

Fig. 5.—Prevention by glucose of inactivation of the formic hydrogenlyase of *A. polymyxa* by dilution.

An explanation for the difference in stability of suspensions of *E. coli* and *A. polymyxa* was sought in the relative rates of oxidation of a substrate, such as glucose, by oxygen. Cell suspensions of *A. polymyxa* and *E. coli,* having comparable rates of production of hydrogen from formate, were tested for rate of uptake of oxygen in the presence of 0.1M glucose and for the rate of reduction of methylene blue by this substrate using the Thunberg technique. From these results it was possible to calculate that a suspension of *A. polymyxa* capable of reducing methylene blue at the same rate as a suspension of *E. coli* would absorb oxygen in the presence of glucose at about 50 times the rate for the corresponding suspension of *E. coli.* Potentiometric estimation of the redox potential of anaerobic suspensions of *A. polymyxa* on addition of glucose, glycerol, pyruvate, or ethanol showed a rapid fall in potential to values below —0.200 V. in each instance. Suspensions of *E. coli* gave rather lower potential readings.

(*d*) *Reagents Inhibiting the Formic Hydrogenlyase System of* A. polymyxa

Glutamate caused a rapid reduction of redox potential but was relatively ineffective as a stabilizing agent. However, on adding glutamate to an active cell suspension it was found to be inhibitory at a concentration of 0.05M and this property was shown to be common to a number of dicarboxylic acids (Table 8). It is possible therefore that glutamate stabilizes the formic hydrogenlyase system by reducing the redox potential of the cell suspension, its effect being masked by its direct inhibitory effect on the enzyme system.

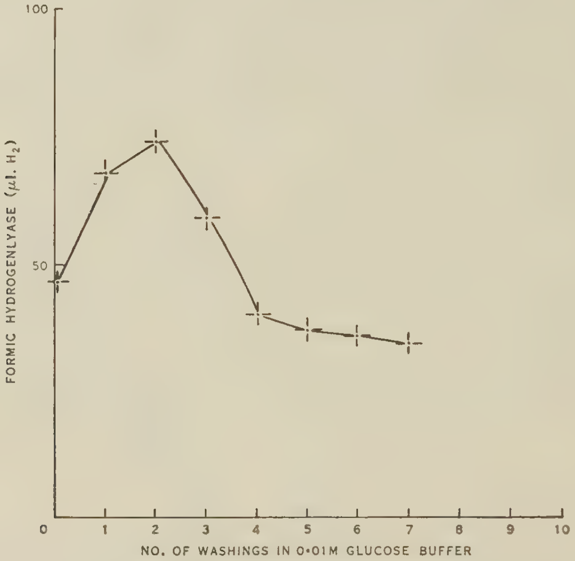

Fig. 6.—Effect on the formic hydrogenlyase activity of *A. polymyxa* of successive washing in buffer containing 0.01M glucose.

Table 7

STABILIZATION OF FORMIC HYDROGENLYASE OF *A. POLYMYXA* BY SULPHITE TOGETHER WITH HAEMATEIN

1.0 ml. twice-washed cell suspension, 1.0 ml. of stabilizing solution, 0.5 ml. 1M formate

Addition	Activity (% of activity of once-washed cells)
Water	Nil
Na_2SO_3 1·0%	13
Haematein 0·01%	Nil
Haematein 0·01%+Na_2SO_3	39
Haematein 0·01%+Na_2SO_3 0·01% ..	26
Haematein 0·0001%+Na_2SO_3 1·0% ..	10
Haematein 0·0001%+Na_2SO_3 0·01% ..	6

The anomalous effect of high concentrations of glucose on the production of hydrogen from formate, apparent in Table 6, has been further investigated by estimating the production of hydrogen by washed cell suspensions of *A. polymyxa* from formate, glucose, and mixtures of the two substrates of various concentrations. Hydrogen was evolved rapidly from each substrate but a mixture containing 1.0M concentrations of both substrates failed to cause

TABLE 8

INHIBITION OF HYDROGEN PRODUCTION FROM FORMATE BY DICARBOXYLIC ACIDS, USING A WASHED-CELL SUSPENSION OF *A. POLYMYXA*

Inhibitor (final concentration 0·05M)	H_2 Evolved in 30 Min. (cu. mm.)	Inhibition (%)
Water	127	Nil
Glutamate	91	28
Glutarate	55	56
Aspartate	7	94
Succinate	13	90
Fumarate	47	63
Malate	46	64
Maleate	Nil	100
Oxalate	21	84

TABLE 9

PRODUCTION OF HYDROGEN BY *A. POLYMYXA* FROM MIXTURES OF GLUCOSE AND FORMATE

Final Substrate Concentrations	H_2 Evolved in 30 Min. (cu. mm.)	H_2 Evolved (% of H_2 evolved from formate)
Formate* 1·0M	195	100
Glucose 1·0M	95	—
Formate 1·0M+glucose 1·0M	Nil	Nil
Formate* 0·5M	137	100
Glucose 0·5M	75	—
Formate 0·5M+glucose 0·5M	17	12
Formate* 0·2M	57	100
Glucose 0·2M	100	—
Formate 0·2M+glucose 0·2M	26	45
Formate 0·2M+glucose 0·06M	52	91
Formate 0·2M+glucose 0·02M	56	98
Formate 0·2M+glucose 0·006M	55	97

* Results using different cell suspensions.

gas production (Table 9). With 0.2M formate as substrate, concentrations of glucose greater than 0.02M were inhibitory.

TABLE 10

INACTIVATION OF THE FORMIC HYDROGENLYASE SYSTEM OF *A. POLYMYXA* BY OXIDATION AND REDUCTION

Inhibitor	Final Concentration	Inhibition (%)
Water	-	Nil
$KMnO_4$	0·002M	23
H_2O_2	0·02M	87
H_2O_2	0·02M+0·005M Na_2SO_3 after 30 min. at 30°C.	67
Na_2SO_3	0·005M	51
H_2O_2	0·02 Vol.+0·005M $Na_2S_2O_4$ after 30 min. at 30°C.	88
$Na_2S_2O_4$ ..	0·005M	87

In conformity with the inactivation of the formic hydrogenlyase system of bacteria by oxygenation, permanganate, and hydrogen peroxide were found to be effective inhibitors (Table 10). The effect of peroxide was partially reversed by sulphite, but not by hydrosulphite, which was itself strongly inhibitory. Sulphite was also an inhibitor but its effect was less marked.

TABLE 11

INHIBITION OF THE FORMIC HYDROGENLYASE OF *A. POLYMYXA* BY REAGENTS REACTING WITH SULPHYDRYL GROUPS

Inhibitor		H_2 Evolved in 30 Min. (cu. mm.)	Inhibition (%)
Water		103	Nil
p-Aminophenylarsenate			
	0·04M	21	79
	0·002M	73	29
Iodoacetate	0·04M	5	95
	0·002M	31	70
Iodoacetamide	0·04M	Nil	100
	0·002M	11	89
Iodosobenzoate	0·04M	Nil	100
	0·002M	55	46

Reagents reacting with sulphydryl groups have also proved to be inhibitory (Table 11), iodoacetamide causing complete inhibition at a concentration of 0.04M.

The chelating agents, cupferron, 8-hydroxyquinoline, dimethyl glyoxime, *aa'*-dipyridyl, and various derivatives of 8-hydroxyquinoline have been shown to cause significant inhibition at concentrations of 0.004M, cupferron causing complete inhibition at a concentration of 0.004M (Table 12). However, 2-hydroxyquinoline, which does not chelate with metals, is also inhibitory at equivalent concentrations.

Of reagents known to form complexes with haem compounds, sodium cyanide, sodium fluoride, hydroxylamine, and sodium azide were inhibitory, the approximate concentrations giving 50 per cent. inhibition being 10^{-5}M, 2×10^{-3}M, 10^{-4}M, and 10^{-4}M respectively.

Table 12

INHIBITION OF THE FORMIC HYDROGENLYASE OF *A. POLYMYXA* BY REAGENTS CHELATING WITH FERROUS IONS

Inhibitor	Inhibition (%)
Water	Nil
2-Hydroxyquinoline 0·00004M	24
8-Hydroxyquinoline 0·00004M	77
8-(OH)quinoline 5:sulphonic acid 0·004M	2
8-(OH)quinoline 5:sulphonic acid 0·00004M	Nil
8-(OH)7:iodoquinoline 5:sulphonic acid 0·004M	76
8-(OH)7:iodoquinoline 5:sulphonic acid 0·00004M	2
8-(OH)dibromoquinoline 5:sulphonic acid 0·004M	46
8-(OH)dibromoquinoline 5:sulphonic acid 0·00004M	12
Dimethylglyoxime 0·004M	68
Dimethylglyoxime 0·00004M	Nil
aa'-Dipyridyl 0·004M	76
aa'-Dipyridyl 0·00004M	34
Cupferron 0·004M	100
Cupferron 0·00004M	22

IV. Discussion

It is evident that several factors affect the stability of the formic hydrogenlyase and related enzyme systems of *A. polymyxa* and other bacteria. The influence of dilution on stability can be related to three factors: (1) the removal of residual glucose and other oxidizable metabolites from the cells, (2) the addition of buffer containing dissolved oxygen, and (3) the possible removal of diffusible coenzymes. That the two first factors are of importance to the stability of the hydrogenlyase and hydrogenase systems is evident from Tables 1-7 and Figures 1, 2, and 5. The leaching out of coenzymes from the cells probably plays little part in the dilution effect but the progressive inactivation of cells repeatedly washed with glucose buffer suggests that inactivation of the enzyme system responsible for the production of hydrogen from formate can be brought about by some such process.

The evidence suggests that many of the enzyme systems concerned with hydrogen transfer are readily inactivated by oxidation, the ease of oxidation varying considerably. At a certain oxidation-reduction level some of the enzymes may be active, others inactive, and some partially active. If it is assumed that production of hydrogen from substrates such as formate, glucose, or pyruvate takes place as a result of the activation of the substrate by its specific dehydrogenase, which passes on hydrogen or electrons to the hydrogenase system, the results obtained in this and previous studies can readily be explained in terms of the relative ease of oxidation of the dehydrogenases and hydrogenase. Thus it has been shown that the formic dehydrogenase is more readily oxidized than the pyruvic dehydrogenase or hydrogenase, and these enzymes are more easily inactivated by oxidation than the glucose dehydrogenase system. Unless therefore the amounts of the various enzymes present in the cells are of different orders of magnitude in terms of hydrogen transport, it may be expected that the formic hydrogenlyase system would be inactivated in parallel with the formic dehydrogenase, and that the glucose hydrogenlyase system would be more resistant to oxidation and be inactivated in parallel with hydrogenase. Figures 3 and 4 show this to be so. De Ley (1951) has demonstrated a similar relationship between the activity of the formic hydrogenlyase and hydrogenase in cells of *E. coli* grown on nitrogen-deficient media.

Hinshelwood (1946) has pointed out that in a system consisting of two or more consecutive enzymes, no one enzyme will be solely rate-determining, and it follows therefore that a rectilinear relationship between hydrogenase and glucose hydrogenlyase would not be expected over the whole range of activities. The proportionality between formic dehydrogenase and formic hydrogenlyase over the major portion of the range of activities tested would indicate therefore that formic dehydrogenase was considerably less active, in terms of hydrogen transport, than the hydrogenase of *E. coli*. The deviation of the relationship between the glucose hydrogenlyase and hydrogenase of *A. polymyxa* from a straight line indicates that the hydrogenase is at least comparable in activity to the glucose dehydrogenase system and may in fact be more active.

In an aerobic system consisting of a dehydrogenase with its substrate, cytochrome oxidase, and the cytochromes and flavoproteins, the mean Eh would depend on the concentration of substrate and dehydrogenase, on the E_0 value of the system "substrate-oxidized substrate," on the concentration of the cytochrome-cytochrome oxidase and flavoproteins, and on the partial pressure of oxygen. A system having a low ratio of dehydrogenase activity to total oxidase activity in the presence of a certain concentration of substrate would require a relatively low partial pressure of oxygen for the maintenance of a particular Eh level. On the other hand a system having a high ratio of dehydrogenase to oxidase would require a high partial pressure of oxygen to maintain this Eh in the presence of an equal concentration of substrate. Stahly and Werkman (1942) showed that during metabolism of glucose solutions by aerated cultures of *A. polymyxa*, the Eh of the medium was maintained at a low level if the glucose concentration remained high. As the glucose concentration fell the Eh of the system increased, even in the absence of forced aeration.

Since the ratio of dehydrogenase activity to oxidase activity for the strain of *E. coli* used in these experiments was approximately 50 times the corresponding ratio for strain GO(7) of *A. polymyxa*, the different behaviour of suspensions of these organisms on washing or dilution may be explained in terms of the resulting higher Eh of a suspension of *A. polymyxa* as compared with a suspension of *E. coli,* under similar conditions of glucose concentration and partial pressure of oxygen. This would lead to more extensive oxidative inactivation of the enzymes producing molecular hydrogen in cells of *A. polymyxa* than in cells of *E. coli.*

A reasonable explanation is provided by the above hypothesis for the different concentrations of ferrous salts required in the growth medium (Crewther 1953) for the production of washed suspensions of *A. polymyxa* having active hydrogenlyase systems utilizing formate, pyruvate, and glucose and an active hydrogenase system. The order of concentration of $FeSO_4.7H_2O$ required to give active washed-cell suspensions and the lability of the various enzyme systems is: glucose hydrogenlyase = hydrogenase < pyruvic hydrogenlyase < formic hydrogenlyase. The presence of a sludge of insoluble ferrous salts in the bacterial suspension would not only retain a certain amount of glucose, but it would also deplete the dissolved oxygen in forming the corresponding ferric salts.

The data presented here also support the thesis of Stephenson and Stickland (1932) that hydrogen is produced from glucose by a mechanism which does not involve its conversion to formate as an intermediate. Thus the inactivation of glucose hydrogenlyase by oxidation is more closely related to the inactivation of hydrogenase than it is to that of formic hydrogenlyase, and it is therefore possible to obtain cells of either *A. polymyxa* or *E. coli* with active glucose hydrogenlyase systems but with the formic hydrogenlyase system at levels which cannot be estimated. The mutual inhibition of hydrogen production from formate by glucose and vice versa (Table 9) would also suggest that the two systems differ considerably. It is difficult to conceive of a mechanism for this inhibition and it may well be concerned with changes in the permeability of the cells.

In interpreting the results obtained in these experiments it has been assumed that the major effect of glucose is to prevent oxidation of the enzyme systems. It is difficult in such experiments to distinguish between stabilization and reactivation, and this question will be considered again in later publications.

The nature of the oxidation, and whether the enzymes themselves are oxidized cannot be determined from the data. The inactivation of the formic hydrogenlyase system by sulphydryl reagents may be due to the presence of an essential SH group on one of the enzyme components of the system (Table 11); on the other hand the demonstration by Fowler (1951) that 2,4-dinitrophenol prevents *E. coli* from changing from an oxidative metabolism to a fermentative metabolism and vice versa, and the demonstration that diphosphopyridine nucleotide is dephosphorylated much more readily by a specific enzyme of brain when it is in the oxidized form (McIlwaine 1949) may be related to the phenomenon under consideration. A specific hydrogen carrier essential to the production of hydrogen from formate or glucose may be rendered more labile to hydrolytic attack by conversion to the oxidized form. The phenomenon is probably similar to that observed for *Cl. acetobutylicum* by Davies (1942) and for a strain of *E. coli* by Lascelles (1950). In each instance glucose stabilized the enzyme systems producing hydrogen from glucose and formate respectively, and exposure to air or oxygen hastened the inactivation process.

Yudkin (1932) observed that aeration of cultures of *E. coli* during growth produced cells with reduced, or in some cases no hydrogenlyase activity, depending on the presence and nature of carbohydrate in the medium. The presence in the growth medium of hydrogen acceptors, such as nitrate, also led to decreased activity, and it seems probable that the effective redox potential of the culture determines the final activity of the cells. In addition Yudkin commented on the absence of hydrogenlyase activity from washed suspensions of *E. coli* grown on an inorganic medium containing glucose, although hydrogen was produced during growth of the cells on the medium. The same strain of *E. coli* was found to produce an active washed suspension when grown on a peptone glucose medium. In terms of the above hypothesis, it is possible that peptone contains components which stabilize the formic hydrogenlyase against oxidation or against inactivation under oxidizing conditions.

The evidence presented above for the inactivation of the hydrogenlyase systems of *A. polymyxa* by oxidation is supported by the inactivation of formic hydrogenlyase in the presence of permanganate and hydrogen peroxide and the partial reversal of this process by sulphite (Table 10). There can be little doubt that an organic iron compound also constitutes an important part of the hydrogenlyase system since all reagents known to react with such compounds are strongly inhibitory. The inhibition by hydrosulphite (Table 10) may in fact be due to its ability to reduce coordinated ferric compounds to the ferrous state.

Of the chelating and related compounds listed in Table 12, all but 2-hydroxyquinoline and dimethylglyoxime have been shown by Albert and Gledhill (1947) and Albert and Magrath (1947) to chelate with ferrous or ferric ions under physiological conditions. It may be significant that these two compounds and the sulphonated quinolines which form soluble complexes with ferrous ions are less effective inhibitors than 8-hydroxyquinoline, αα′-dipyridyl, and cupferron, which form insoluble complexes. The iron of iron-porphyrin compounds in the ferric state contains only one easily substituted group and cannot form chelate complexes; ferrous or some other metal ion may therefore be required for activity of the formic hydrogenlyase system. However, since 2-hydroxyquinoline and dimethyl glyoxime show appreciable inhibition, and as all the reagents possess certain groups in common and like pyridine may combine with iron compounds such as the iron-porphyrins, this interpretation is open to considerable doubt. The suggestion that the inhibition of cell-free formic hydrogenlyase by αα′-dipyridyl is due to removal of iron (Gest and Gibbs 1952) is equally equivocal.

No obvious explanation suggests itself for the inhibition of formic hydrogenlyase by dicarboxylic acids. However, if the suggestion that the system contains an essential iron porphyrin or similar moiety is correct (Stephenson 1939; Waring and Werkman 1944; Warburg 1949), assuming the iron to be at least partly in the ferric state, the inhibition may be due to substitution of a hydroxyl group on the iron by the anion of the dicarboxylic acid in a manner resembling the reaction of anions with the prosthetic group of catalase (Agner and Theorell 1946).

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THE ACCUMULATION OF ACETALDEHYDE BY SUSPENSIONS OF YEASTS

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Summary

The sherry flor yeast, *Saccharomyces beticus*, which causes accumulation of acetaldehyde when growing as a film on the surface of alcohol-containing media, also causes an accumulation of acetaldehyde when shaken with ethyl alcohol in air. The ability to accumulate aldehyde under these conditions is not confined to this species, however, but was also found in *S. cerevisiae*, *S. carlsbergensis*, and *S. ellipsoideus*.

Added acetaldehyde is consumed by suspensions of the yeast under both aerobic and anaerobic conditions in the absence of alcohol and under anaerobic conditions in the presence of alcohol.

When suspensions are shaken with mixtures of alcohol and aldehyde under aerobic conditions the yeast tends to bring about an equilibrium between the concentrations of these two components. The value of the equilibrium ratio [alcohol]/[aldehyde] is not constant, but varies with the concentration of alcohol and the temperature, and is also affected by the presence of certain other substrates.

I. Introduction

During the course of a series of investigations carried out by the author (Fornachon 1953) on the metabolism of the sherry flor yeast, *Saccharomyces beticus*, it was found that this yeast causes the accumulation of considerable amounts of acetaldehyde when growing on wine or synthetic culture media containing ethyl alcohol. *Saccharomyces beticus* is a fermentative yeast with a strongly developed film stage of growth. When seeded into grape juice or other suitable media containing fermentable sugar, it brings about a normal alcoholic fermentation, but if the fermented liquid is subsequently allowed to remain under aerobic conditions at a temperature near 20°C. for a few weeks, the yeast then grows as a film or pellicle on the surface of the liquid. During this phase of aerobic film growth, the yeast brings about the oxidation of ethyl alcohol with accumulation of acetaldehyde which is formed as an intermediate. Under certain conditions, the acetaldehyde has been found to reach over 0.05 per cent. in wine and synthetic media containing between 14 and 16 per cent. of alcohol by volume.

It has long been known that yeasts possess the enzyme alcohol dehydrogenase which brings about the reversible dehydrogenation of ethyl alcohol to

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acetaldehyde in the presence of cozymase (co-dehydrogenase I or diphosphopyridine nucleotide = DPN). The reaction is represented by the equation

$$CH_3.CH_2OH + DPN \rightleftharpoons DPNH_2 + CH_3.CHO.$$

Chief interest in this reaction has usually been associated with alcoholic fermentation, where the alcohol dehydrogenase functions as an aldehyde reductase reducing acetaldehyde to ethyl alcohol while the oxidized cozymase resulting from this reaction is again reduced by the dehydrogenation of triose phosphate to phosphoglyceric acid. Although Trillat and Sauton (1910) observed the accumulation of acetaldehyde when aqueous solutions of alcohol were agitated in air with large quantities of yeast cells, the ability of yeasts to bring about such an accumulation seems to have received comparatively little attention. This appears to be due to the fact that the equilibrium in the reaction represented by the equation lies far to the left and to the small concentrations of alcohol commonly used by investigators who have studied the reaction. Thus Green (1940) states that the reaction between the coenzyme and the alcohol system is in favour of the reduction of aldehyde to alcohol and the oxidation of reduced coenzyme. Hence in order to study the aerobic oxidation of alcohol, it is essential to fix the aldehyde with a suitable reagent. A similar view is expressed by McShann (1949).

Gottschalk (1941, 1942) investigating quantitatively the course of acetaldehyde utilization by compressed (baker's) yeast under aerobic and anaerobic conditions in the absence and in the presence of additional substrates obtained the following results:

(*a*) Anaerobically, acetaldehyde undergoes dismutation to ethyl alcohol and acetic acid, a process much enhanced when glucose is simultaneously fermented;

(*b*) Aerobically, aldehyde consumption is much greater than under anaerobic conditions, and here again consumption is increased by the presence of glucose. Under optimal conditions of aeration, acetaldehyde is oxidized by compressed yeast at the same rate as glucose; the oxidation of 1 mole of acetaldehyde to CO_2 and H_2O is accompanied by the reduction of another mole of acetaldehyde to ethyl alcohol according to the equations

$$CH_3.CHO + 2O_2 + H_2O + DPN \rightarrow DPNH_2 + 2CO_2 + 2H_2O$$
$$CH_3.CHO + DPNH_2 \rightarrow DPN + CH_3.CH_2OH.$$

From these data he concluded that the rate of the reaction between reduced cozymase and acetaldehyde (activated by alcohol dehydrogenase) greatly exceeds the rate of the reaction in which the reoxidation of reduced cozymase is effected by the flavoprotein-carrier-oxygen system. He found that yeast cells in contact with a mixture of acetaldehyde, ethyl alcohol, and acetic acid under aerobic conditions attack at first and selectively the acetaldehyde; down to very low concentrations the aldehyde remains the only substrate for the respiratory system of the yeast.

In view of these findings it is somewhat surprising to find that acetaldehyde accumulates to the extent of over 0.05 per cent. under the influence of the sherry flor yeasts in growing cultures. It was therefore considered appropriate to examine further certain aspects of the accumulation of aldehyde by suspensions of intact yeast cells.

II. Materials and Methods

The yeasts used in these studies were:

Saccharomyces beticus	Strain 81 of the Waite Institute collection,
S. cerevisiae	Strain 190 of the Waite Institute collection,
S. carlsbergensis	Strain 191 of the Waite Institute collection,
S. ellipsoideus (port strain)	Strain 139 of the Waite Institute collection,
S. ellipsoideus (champagne strain)	Strain 138 of the Waite Institute collection.

The yeasts were grown for 48 hr. in 20-oz. flat bottles, each containing 100 ml. of diluted grape juice inoculated with 10 ml. of a 24-hr. culture in similar medium and incubated at 25°C. During incubation the bottles lay flat in the incubator and were frequently shaken by hand.

To prepare suspensions of the yeasts, the 48-hr. cultures were centrifuged and the cells washed three times in sterile tap water and finally resuspended as a 10 per cent. w/v suspension in sterile 0.05M phosphate buffer at pH 3.25. The yield obtained was about 2 g. wet yeast from 100 ml. of culture. The suspensions were stored in the refrigerator till required but were never more than 48 hr. old when used. The phosphate buffer was prepared by adding 5 ml. of 0.05M H_3PO_4 to 100 ml. of 0.05M KH_2PO_4 solution. The value of 3.25 was chosen for the pH of the buffer because it falls within the range of the pH values commonly found in the wines used as flor sherry base.

The diluted grape juice was prepared as follows:

300 ml. of pasteurized grape juice (pH about 3.5 and containing about 24 per cent. reducing sugar) was diluted to 1 l. with distilled water. Then 4.5 g. of $(NH_4)_2SO_4$, 0.75 g. K_2HPO_4, and 0.1 g. $MgSO_4.7H_2O$ were added. The medium was dispensed in tubes and bottles as required, and sterilized in the steamer.

Acetaldehyde used in the experiments was distilled from aldehyde ammonia prepared in the laboratory and was kept in the refrigerator as a 1 per cent. aqueous solution. The solution was redistilled a few hours before use and the acetaldehyde content of the distillate determined.

Experiments were carried out with yeast suspensions added to substrates in 0.05M phosphate buffer in 8-oz. flat bottles. The total volume of suspension and substrate in each bottle was 10 ml. For experiments with an atmosphere of air, the bottles were closed with screw-on caps fitted with rubber linings, and when other gas mixtures were used, the bottles were closed with rubber bungs pierced by capillary tubing carrying stopcocks. During experiments the bottles were placed horizontally on an electric shaking machine and were shaken at 120 oscillations per minute with an amplitude of 4 cm. The whole apparatus was placed in an electric incubator and temperature variation did not exceed ± 0.5°C. during any one experiment.

Analysis of samples of gas drawn from several bottles at the end of experiments showed that the oxygen content of the atmosphere in the bottles decreased only very slightly during an experiment.

At the end of an experiment the reaction was stopped by the addition of 1 ml. per bottle of a saturated solution of $AgSO_4$ as recommended by Janke and Kropacsy (1935). The contents of the bottles were then chilled with crushed ice and centrifuged and the supernatant liquid was decanted and examined.

TABLE 1

EFFECT OF ALCOHOL CONCENTRATION ON ACCUMULATION OF ALDEHYDE IN SUSPENSIONS OF *S. BETICUS*

Yeast 200 mg.; pH 3.25; temperature 18°C.; atmosphere air; time 3 hr.; total volume of liquid 10 ml.

Suspension No.	Alcohol Added		Aldehyde Found (mg./10 ml.)
	Vol. %	Mg./10 ml.	
I	0·016	1·3	<0·10
	0·08	6·4	0·10
	0·8	64	0·65
	4·0	320	2·47
II	1·0	79	0·82
	5·0	400	2·34
	10	790	4·20
	15	1200	5·15
III	15	1200	4·93
	20	1600	4·96
	25	2000	2·21
	30	2400	0·50
IV	12	950	4·86
	14	1200	4·95
	16	1300	5·27
	18	1400	5·40
	20	1600	4·84
	22	1800	3·60
	24	1900	2·53
	26	2100	1·92

Aldehyde was distilled from duplicate 2-ml. portions of the liquid in an all-glass still, care being taken to keep the end of the condenser below the surface of the liquid in the receiving flask, which was surrounded by crushed ice. Aldehyde was determined by an adaptation of the bisulphite method of Jaulmes and Espezel (1935), using one-fifth the original quantities of reagents and titrating with 0.02N iodine from a microburette.

Blank experiments were carried out both without yeast cells and with yeast cells and $AgSO_4$ added at the beginning of the experiments. The results of these blank tests showed that under the experimental conditions, non-enzymic oxidation of ethyl alcohol to acetaldehyde produced 0.1 mg. of aldehyde from 10 ml. of 14 per cent. alcohol in 3 hr. Determinations of aldehyde production and aldehyde consumption by suspensions of yeasts always included control tests in which the substrates were shaken without yeast cells and appropriate corrections were applied to the analytical results.

III. Experimental Results

(*a*) *Effect of Alcohol Concentration on Aldehyde Accumulation*

The amounts of aldehyde that accumulated in suspensions of S. *beticus* when shaken in different concentrations of alcohol under aerobic conditions for 3 hr. are shown in Table 1.

It can be seen that the accumulation of aldehyde is dependent on the concentration of alcohol; it increased markedly with increased concentrations of alcohol and under these conditions greatest accumulation occurred near 18 per cent. of alcohol. When the alcohol was raised above 20 per cent., however, the accumulation of aldehyde was greatly depressed.

(*b*) *Accumulation of Aldehyde by Different Yeasts*

Although these investigations have been chiefly concerned with S. *beticus*, suspensions of several other yeasts have also been prepared and tested. The results of such tests are shown in Table 2, from which it can be seen that although the yeasts vary in their ability to accumulate aldehyde under these conditions, the characteristic is not confined to the sherry flor yeast.

Table 2

ALDEHYDE ACCUMULATION BY SUSPENSIONS OF DIFFERENT YEASTS IN 10 PER CENT. ALCOHOL BY VOLUME

Yeast 200 mg.; pH 3.25; temperature 20°C.; atmosphere air; time 3 hr.; total volume of liquid 10 ml.

Yeast	Aldehyde (mg./bottle)
S. beticus	4·62
S. cerevisiae	2·54
S. carlsbergensis	3·41
S. ellipsoideus (port strain)	3·52
S. ellipsoideus (champagne strain)	3·88

(*c*) *Effect of pH on Accumulation of Aldehyde*

Adjusting the pH of the suspensions at various levels over the range from 2.0 to 7.8 had little effect on the accumulation of aldehyde. At pH values of 2.0 and 2.5 the amount of aldehyde was slightly less than at pH 3.0, but no significant differences were found over the range from pH 3.0 to 7.8. The most likely explanation of this result would seem to be that the pH in the immediate environment of the enzyme systems, that is, of the contents of the yeast cells, was not significantly affected during the experiment by such changes in the pH of the suspending medium. It has been shown by Gottschalk (1943, 1949) that the pH of the yeast cell is strongly buffered at about pH 6, and is not readily altered by changes in the pH of the surrounding medium.

TABLE 3

ALDEHYDE ACCUMULATION BY *S. BETICUS* UNDER DIFFERENT MIXTURES OF AIR AND NITROGEN

Yeast 200 mg.; pH 3.25; temperature 18°C.; alcohol concentration 14 vol. per cent.; time 3 hr.; total volume of liquid 10 ml.

Bottle No.	Treatment	Oxygen in Gas Phase (%) (calculated)	Aldehyde Formed (mg./bottle)
1	Atmospheric air	21	4·55
2			4·37
3	Evacuated to 250 mm. Hg and refilled with N_2 once	7	4·06
4			4·13
5	Evacuated to 125 mm. Hg and refilled with N_2 once	3·5	3·67
6			3·68
7	Evacuated to 125 mm. Hg and refilled with N_2 three times	0·1	1·20
8			1·34
9	Evacuated to 75 mm. Hg and refilled with N_2 five times	None	<0·1
10			<0·1

(*d*) *Effect of Oxygen Tension on Accumulation of Aldehyde*

In order to carry out experiments under different partial pressures of oxygen, the bottles were evacuated to predetermined pressures and refilled with nitrogen purified by passage through three scrubbers of alkaline pyrogallol and allowed to stand over alkaline pyrogallol. It is not, of course, claimed that the oxygen tension in the bottles could be accurately controlled by such means, but it was possible to achieve a range between aerobic and anaerobic conditions. The effects of such treatments on the accumulation of aldehyde by *S. beticus* in 14 vol. per cent. alcohol are shown in Table 3.

In the experiment represented by the results in Table 3, aldehyde accumulation was depressed by each reduction in oxygen tension, but in other experiments the treatment given to bottles 3 and 4 in Table 3 has had no significant effect on the amount of aldehyde found. However, aldehyde accumulation has always been much depressed at the lower oxygen tensions and under anaerobic conditions no accumulation of aldehyde could be detected.

(*e*) *Consumption of Acetaldehyde by the Yeast*

Not only do the cells fail to accumulate aldehyde under anaerobic conditions, but if aldehyde is added at the beginning of the experiment, some of it disappears and the volatile acid increases by an amount equivalent to slightly less than half of the aldehyde consumed. In an experiment using 400 mg. of yeast cells to increase the speed of the reaction and an initial aldehyde concentration of 9.5 mg. per bottle, 5.3 mg. (0.121 millimoles) of aldehyde were consumed, and 3.5 mg. (0.058 millimoles) of acetic acid were formed. These data are in accordance with the results of Gottschalk (1941, 1942) indicating that the chief mechanism by which yeasts metabolize aldehyde under anaerobic conditions in the absence of other substrates is dismutation to acetic acid and ethyl alcohol.

TABLE 4

CONSUMPTION OF ALDEHYDE UNDER DIFFERENT CONDITIONS BY *S. BETICUS*

Yeast 200 mg.; pH 3.25; temperature 20°C.; time 3 hr.; total volume of liquid 10 ml.

Substrate	Aldehyde Metabolized (mg.)	
	Aerobically	Anaerobically
Aldehyde 9·5 mg.	3·8	2·7
Aldehyde 9·5 mg. + 20 mg. glucose	5·7	4·8
Aldehyde 9·5 mg. + 12 mg. acetate	2·5	1·8
Aldehyde 9·5 mg. + 3·5 ml. of 40% alcohol (14 vol. % alcohol)	0·0	0·7

Experimental study of the effects of several factors on the consumption of aldehyde by the yeast have shown that in the absence of alcohol, aldehyde is consumed more rapidly aerobically than anaerobically (see Gottschalk 1941), but in 14 per cent. alcohol the reverse is true. In fact, in the presence of alcohol, aldehyde consumption under aerobic conditions is only apparent if the initial concentration of aldehyde is above a certain minimum value. As will be shown later, this minimum value varies with the alcohol concentration.

In Table 4 are shown the amounts of aldehyde consumed by suspensions of S. *beticus* in the presence and absence of other substrates and under atmospheres of air and of nitrogen. As can be seen, aldehyde consumption was increased by the presence of glucose as was shown previously by Gottschalk (1942), but it was depressed by acetate or alcohol. Anaerobic conditions depressed aldehyde consumption except in the presence of alcohol.

When other substrates were added to alcohol-aldehyde mixtures before treatment with the yeast cells, the results shown in Table 5 were obtained. Since these experiments were carried out with several different suspensions, the results are expressed as increased consumption as compared with controls in which aldehyde and alcohol were the only substrates. Galactose, glycerol, lactate, malate, tartrate, citrate, and succinate were also tested but were without effect on aldehyde consumption under these conditions.

As can be seen from Tables 4 and 5, glucose increased the consumption of aldehyde less in the presence of alcohol than in its absence but acetate, which depressed aldehyde consumption in the absence of alcohol, actually caused increased consumption in the presence of alcohol.

TABLE 5

EFFECT OF OTHER SUBSTRATES ON THE CONSUMPTION OF ALDEHYDE BY *S. BETICUS* IN THE PRESENCE OF 14 PER CENT. ALCOHOL

Yeast 200 mg.; pH 3.25; temperature 20°C.; time 3 hr.; initial aldehyde 9.5 mg.; total volume of liquid 10 ml.

Additional Substrate	Increased Aldehyde Consumption (mg.)	
	Aerobically	Anaerobically
Glucose 20 mg.	1·2	1·6
Glucose 40 mg.		2·5
Glucose 60 mg.		2·6
Acetate 12 mg.	1·2	1·2
Fumarate 20 mg.		0·9

(*f*) *The Alcohol-Aldehyde Equilibrium*

When the yeast cells were shaken with mixtures of alcohol and aldehyde under aerobic conditions, it was noticed that the aldehyde sometimes increased and sometimes decreased in amount, depending on the initial concentration of aldehyde present. This suggested that the aldehyde concentration tended to reach an equilibrium value for any particular set of conditions. Accordingly a series of experiments was carried out with varied amounts of aldehyde. In each experiment the alcohol content of all bottles was the same and different amounts of aldehyde were introduced. The yeast cells were then added, and after the bottles had been shaken for 3 hr. the reaction was stopped and the changes in aldehyde content during the experiment were determined. It was found that aldehyde accumulated in bottles with a low initial aldehyde content, whereas it decreased in those with a high initial content, but somewhere in between was a point of equilibrium at which the aldehyde content remained unchanged during the experiment. The data presented in Table 6 are typical of such experiments and indicate the equilibrium aldehyde concentration in 10 volume per cent. alcohol at 25°C.

If the change in aldehyde content is plotted against the initial aldehyde content, then it is found that the data presented in Table 6 indicate that zero change would occur when the initial aldehyde content was about 7.65 mg. per bottle, a concentration of about 0.0174M.

The concentration of alcohol was not determined at the end of each of the experiments represented by the data in Tables 6 and 7. However, in two experiments suspensions of the yeast were shaken with 10 per cent. and with 15 per cent. by volume of alcohol for 3 hr. and the alcohol contents of the suspensions were then determined by the dichromate method of Joslyn and Amerine (1941). It was found that the loss of alcohol during the experiments was too small to be detected by this method, the results of which were reproducible to within ± 0.1 per cent. of alcohol by volume. In four experiments samples of the gas in the bottles were collected after 3 hr. and analysed for oxygen and carbon dioxide. The greatest amount of carbon dioxide found was 5.7 mg. per bottle, corresponding to complete oxidation of about 3 mg. of alcohol. Although the error involved in such measurements is admittedly large, it is considered that these, together with the alcohol determinations mentioned above, justify the assumption that the alcohol did not change significantly during the course of the experiments under consideration, in which the changes in acetaldehyde concentration were quite small (Table 6). Accordingly the amounts of alcohol added at the beginning of experiments, and expressed in terms of molar concentrations, have been used in calculating the values of [alcohol]/[aldehyde] at equilibrium. For the data recorded in Table 6 this value is 99.

TABLE 6

EFFECT OF INITIAL ALDEHYDE CONCENTRATION ON ALDEHYDE ACCUMULATION AND CONSUMPTION BY *S. BETICUS* UNDER AEROBIC CONDITIONS

Yeast 200 mg.; pH 3.25; temperature 25°C.; atmosphere air; time 3 hr.; alcohol concentration 10 vol. per cent.; total volume of liquid 10 ml.

Aldehyde (mg. per bottle)		
Initial	Final	Change
4·05	6·10	+2·05
5·25	6·42	+1·17
6·46	6·99	+0·53
7·68	7·66	−0·02
8·90	8·45	−0·45
10·10	9·30	−0·80

Similar experiments have been carried out with different concentrations of alcohol and at different temperatures and the results of these experiments are summarized in Table 7.

It can be seen from Table 7 that the alcohol/aldehyde equilibrium is affected by both temperature and alcohol concentration. The data presented in Table 5, which show that aldehyde consumption in the presence of alcohol and under aerobic conditions is increased by the presence of some other substrates, suggests that these other substrates also affect the equilibrium. In a wine containing 14 per cent. of alcohol by volume and treated with suspensions of *S. beticus,* an alcohol/aldehyde equilibrium was found when the value of [alcohol]/[aldehyde] was 178.

TABLE 7

EFFECT OF ALCOHOL CONCENTRATION AND OF TEMPERATURE ON THE VALUE OF THE EQUILIBRIUM RATIO [ALCOHOL]/[ALDEHYDE] IN SUSPENSIONS OF *S. BETICUS*

Yeast 200 mg.; pH 3.25; atmosphere air; time 3 hr.; total volume of liquid 10 ml.

Temperature (°C.)	Alcohol Concentration			Aldehyde at Equilibrium		[Alcohol]/ [Aldehyde]
	Vol. %	Mg./10 ml.	Molarity	Mg./10 ml.	Molarity	
15	5·0	400	0·87	4·7	0·011	79
25	5·0	400	0·87	4·4	0·010	87
15	8·0	640	1·4	7·1	0·016	87
20	8·0	640	1·4	6·7	0·015	93
25	8·0	640	1·4	6·4	0·015	93
15	10·0	790	1·7	8·4	0·019	89
25	10·0	790	1·7	7·7	0·018	94
15	16·0	1270	2·8	12·5	0·028	100
25	16·0	1270	2·8	11·4	0·026	108

IV. DISCUSSION

It is evident that the accumulation of large amounts of acetaldehyde in some of the experiments with suspensions of yeast cells reported here is associated with the high concentrations of alcohol used. Accumulation of the aldehyde formed as an intermediate in the aerobic oxidation of ethyl alcohol by the yeast must depend on the relative rates of the aldehyde-forming and aldehyde-consuming reactions. Acetaldehyde is formed by the reaction between ethyl alcohol and cozymase activated by alcohol dehydrogenase, but the further oxidation of the aldehyde also involves reactions in which cozymase takes part (Gottschalk 1942). It is to be expected therefore that the distribution of cozymase between the alcohol- and aldehyde-oxidizing systems is influenced by the concentrations of these substrates and that a high concentration of alcohol will increase the accumulation of the acetaldehyde.

One would also expect that, other things being equal, those strains of yeasts which dehydrogenate acetaldehyde least rapidly would bring about the greatest accumulation of aldehyde. In this connection it is worthy of note that, whereas Gottschalk (1941) found that baker's yeast consumed 360 per cent. more acetaldehyde under aerobic conditions than under anaerobic conditions, the corresponding increase with S. *beticus* was only about 40 per cent. (Table 4).

It is not surprising that the concentration of acetaldehyde should have been found to approach an equilibrium value in the presence of yeast cells and ethyl alcohol under aerobic conditions, but it is clear that the ratio between the concentrations of the aldehyde and the alcohol at equilibrium is not what would be expected from the equation

$$CH_3.CH_2OH + DPN \rightleftharpoons DPNH_2 + CH_3.CHO,$$

for the published values for the equilibrium constant for this reaction are between 10^3 and 10^5, depending on conditions (Green 1940). This means that for half reduction of the DPN the ratio [alcohol]/[aldehyde] would have these values (i.e. 10^3-10^5), and that the values found in these investigations could be obtained only if the DPN was mainly in the oxidized form. It is known, however, that intact yeast cells possess another mechanism besides the alcohol-aldehyde system for the reoxidation of reduced cozymase. Under aerobic conditions, the cell is able to transfer hydrogen from the reduced coenzyme to molecular oxygen by means of the diaphorase-cytochrome-cytochrome oxidase system. Reoxidation of the reduced cozymase by this mechanism would displace the equilibrium in the above equation towards the right and result in greater accumulation of acetaldehyde or a lower [alcohol]/[aldehyde] ratio. Although Gottschalk (1941, 1942) has shown that the reoxidation of reduced cozymase by the diaphorase-cytochrome-oxygen system is very slow by comparison with the reoxidation by the alcohol-aldehyde system in baker's yeast, it appears that under the conditions of our experiments and also in growing film cultures of the flor yeasts, an oxidative mechanism involving free oxygen is sufficiently active to compete with acetaldehyde in the reoxidation of reduced cozymase and so increase the accumulation of acetaldehyde.

It has been found that increased concentrations of alcohol do not bring about a *proportional* increase in aldehyde accumulation so that the [alcohol]/[aldehyde] ratio at equilibrium rises with increased concentrations of alcohol (Table 7). This may be ascribed to greater displacement of the oxygen-activated system from the reduced cozymase by the higher concentrations of acetaldehyde.

V. ACKNOWLEDGMENT

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STUDIES ON THE NUCLEIC ACIDS OF THE MALARIA PARASITE, *PLASMODIUM BERGHEI* (VINCKE & LIPS)

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Summary

Changes in the nucleic acid content of the solid residue obtained by haemolysing the blood of mice infected with *Plasmodium berghei* have been examined. The residue from blood in which 25 per cent. of the red blood cells were parasitized contained 20-25 times as much ribosenucleic acid (RNA) and 12 times as much desoxyribosenucleic acid (DNA) as the residue from uninfected blood.

Nucleic acid isolated from the malaria parasite had an absorption spectrum similar to that of yeast nucleic acid.

The purine and pyrimidine composition of RNA and DNA isolated from *P. berghei* has been studied by means of paper chromatography. The purine/pyrimidine ratio for parasite RNA was 1.40, compared with a value of 1.35 for yeast RNA. The corresponding ratios for parasite DNA and thymus DNA were 1.04 and 1.12, whereas the value for DNA isolated from mouse leucocytes was 1.17.

I. Introduction

Despite the large amount of research on malaria, the study of the nucleic acids of the malaria parasite appears to have been overlooked. This is the more remarkable in view of the possibility of a relationship between the mode of action of antimalarials and the nucleic acid cycle in the parasite.

By means of a modification of the Feulgen technique, Deane (1945) showed the presence of desoxyribosenucleic acid (DNA) in the malaria parasites *Plasmodium vivax* and *P. knowlesi.* DNA was restricted to the chromatin of the parasite and its concentration varied according to the stage in the asexual cycle of the parasite. Deane, in fact, suggested a cycle in the DNA content of the parasite; she found the maximum amount in the merozoite stage, less in the trophozoite and schizont stages, and the minimum amount in the gametocytes.

Ball (1946) followed the changes in the nucleic acid phosphorus content of red blood cells of monkeys infected with *P. knowlesi,* and calculated that each parasitized red cell contained 13.5 times as much nucleic acid phosphorus as a normal red cell.

The present paper deals with the quantity of ribosenucleic (RNA) and desoxyribosenucleic acids in *Plasmodium berghei* and their purine and pyrimidine composition.

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II. Materials and Methods

The strain of *Plasmodium berghei* (Vincke & Lips) used in these experiments was obtained from Dr. L. G. Goodwin, The Wellcome Research Institute, London. It has been maintained by twice-weekly passage through white mice. Blood was taken from the tail of an infected mouse and injected intraperitoneally into six other mice. Of the mice inoculated, 90 per cent. became infected; those which resisted the first inoculation always succumbed to a second injection. In all instances, infection was fatal and the period elapsing between inoculation and death of the mouse depended upon the number of parasites in the inoculum; the average period was 10 days, but it ranged from 6 to 21 days. If a large dose was injected, a rapid course of infection with less destruction of the red blood cells was insured. A smaller dose resulted in a longer course of infection and a more marked anaemia. Geiman *et al.* (1946) pointed out the advisability of examining blood in which the parasitaemia had not reached the final stage, as the many associated haematological changes were thus avoided. In all experiments, the blood was collected before the degree of parasitaemia exceeded 35 per cent.

The periodicity of *P. berghei* in mice is 24 hr. and schizogeny occurs between 6.00 and 10.00 a.m. (Ramakrishnan and Prakash 1951). Blood was collected between 9.00 a.m. and 11.00 a.m. when the majority of parasites were in the late segmenting or early trophozoite stages, which, according to Deane (1945), are the most Feulgen-positive stages.

Blood was collected by severing the right jugular vein. One part of 4 per cent. sodium citrate in 0.85 per cent. sodium chloride was used as an anticoagulant for 9 parts of blood. As sufficient material could not be obtained from one mouse, the blood from several mice was pooled.

Red blood cell and leucocyte counts were carried out with the aid of a "Spencer Bright Line" haemacytometer. Parasite counts were made on thin films stained with Leishman's stain and enough cells were counted to reduce the error to 10 per cent., according to the formula

$$N = 45.494 \frac{I - P}{P},$$

where N equals the number of cells to be counted, I equals the sample unit, and P equals the number of parasites per sample unit (Gingrich 1932).

A modification of the method of Schneider (1945) was used to determine the nucleic acid content of the blood residue. DNA was determined by the diphenylamine reaction (Dische 1930) as described by Seibert (1940). RNA was estimated by the method of von Euler and Hahn (1940), modified to permit the detection of 10 μg. RNA. The altered procedure consisted of restricting the total volume to 4.5 ml. and reducing the final concentration of ferric chloride to 0.044 per cent., and that of phloroglucinol to 0.028 per cent. The optical density at 550 mμ was measured in the Beckman spectrophotometer and compared with values obtained for standards containing 20, 40, and 60 μg. RNA, prepared at the same time.

Samples of commercial nucleic acid served as standards for absorption measurements and nucleic acid determinations.

Yeast nucleic acid (sodium salt) was purified by the method of Smith and Markham (1950). The purified product was found to contain 9.1 per cent. phosphorus and 15.9 per cent. nitrogen.

Thymus nucleic acid (sodium salt) was freed from protein by chloroform extraction (Sevag, Lackman, and Smolens 1938), and its phosphorus and nitrogen content determined. These were 9.6 per cent. and 15.3 per cent. respectively.

Commercial samples of adenine, guanine, cytosine, uracil, and thymine for the determination of R_F values and extinction coefficients were recrystallized twice. With the exception of adenine, all were found to be pure and their extinction coefficients corresponded with those given by Wyatt (1951*b*). The adenine sample was contaminated with guanine and the extinction coefficient given by Wyatt was used as a reference.

Phosphorus determinations were carried out by the method of Fiske and Subbarow (1925).

III. Procedure and Results

(*a*) *Increase in the Nucleic Acid Content of Blood of Mice Infected with* P. berghei

Microscopic examination of blood from mice infected with *P. berghei* showed not only the presence of parasites but also changes in the numbers of red and white blood cells. Any experiment designed to show an increase in the nucleic acid content of the blood due to the parasites themselves had to allow for these associated haematological changes. The following procedure was therefore adopted:

Table 1

NUCLEIC ACID CONTENT OF MOUSE LEUCOCYTES

	No. of Determinations	Av. No. Leucocytes ($\times 10^{-6}$/ml.)	Av. Nucleic Acid Content (μg.)	Mean Nucleic Acid Content per Leucocyte (μg. $\times 10^{-6}$)
RNA	10	6·79	6·84	1·01
DNA	12	6·79	37·5	5·52

(i) Samples of blood were collected from uninfected mice and the number of leucocytes present was determined. The leucocytes were separated by centrifugation, washed with ice-cold saline, treated with 0.25 per cent. saponin in saline to remove any red blood cells, and washed again with saline. The residue was extracted thrice with ice-cold 10 per cent. trichloracetic acid and then thrice with a 3:1 mixture of alcohol-ether. Finally, the nucleic acids were extracted from the residue with 5 per cent. trichloracetic acid at 90°C. and estimated colorimetrically. The results are given in Table 1.

(ii) Groups of white mice were infected with *P. berghei* and, when the degree of infection of any one group reached the desired level, the blood from those mice was pooled. Blood from uninfected mice was used as a control. The blood was centrifuged, the cells were washed with ice-cold saline, haemolysed with saponin, and the solid residue washed free of haemoglobin with saline. The residue from infected blood contained free parasites, leucocytes, and ghost red blood cells. During washing, the repeated centrifugation caused the parasites and leucocytes to clump together; hence all counts were made prior to haemolysis. The RNA and DNA content of the residue was determined by the procedure used in (i).

By reference to the values given in Table 1 it was possible to estimate the amount of RNA and DNA due to the leucocytes in the residue and hence to calculate the nucleic acid content of the residue from 10^{10} red blood cells. In this way, the increase in RNA and DNA contents due to the parasites was shown (see Tables 2 and 3).

TABLE 2

RIBOSENUCLEIC ACID CONTENT OF RESIDUE FROM PARASITIZED BLOOD

Parasitized R.B.C. (%)	R.B.C. ($\times 10^{-9}$/ml.)	Leucocytes ($\times 10^{-6}$/ml.)	Total RNA Content (μg./ml.)	RNA Content* of Leucocytes (μg./ml.)	RNA Content† of Parasites and Ghost R.B.C. (μg./ml.)	RNA Content of Residue from 10^{10} R.B.C. (μg.)
0	9·90	6·85	16·6	6·9	9·7	9·8
1	7·76	10·00	21·2	10·1	11·1	14·3
6	6·40	12·80	36·4	12·9	23·5	36·8
10	4·74	9·54	55·6	9·6	46·0	98·0
14	3·17	17·50	66·2	17·6	48·6	154·0
24	3·47	14·14	93·1	14·2	78·9	227·0
32	2·17	15·20	125·0	15·3	109·7	505·0

* Calculated from value given in Table 1 for RNA content per leucocyte.

† These values were obtained by subtracting the RNA content of the leucocytes from the total RNA content.

The increase in RNA was approximately twice that in DNA, as may be seen in Figure 1. The value for RNA at a level of parasitaemia of 32 per cent. seemed unduly high and might have been due partly to some associated changes in the blood itself.

The varying number of red and white blood cells in the different samples of infected blood deserves comment, because, to a certain degree, it reflected the rate of increase of the parasites. When the course of infection was slow the increase in leucocytes and the decrease in red blood cells was much greater than when the course of infection was rapid.

(*b*) *Nucleic Acid Content per Parasite*

As the number of parasites in the residue was known, and the amount of nucleic acid due to the ghost red cells was known from the control experiments, it was possible to determine, from the results shown in Tables 2 and 3, an approximate value for the RNA and DNA content of a single parasite (Tables 4 and 5). These calculations were confined to experiments in which the degree

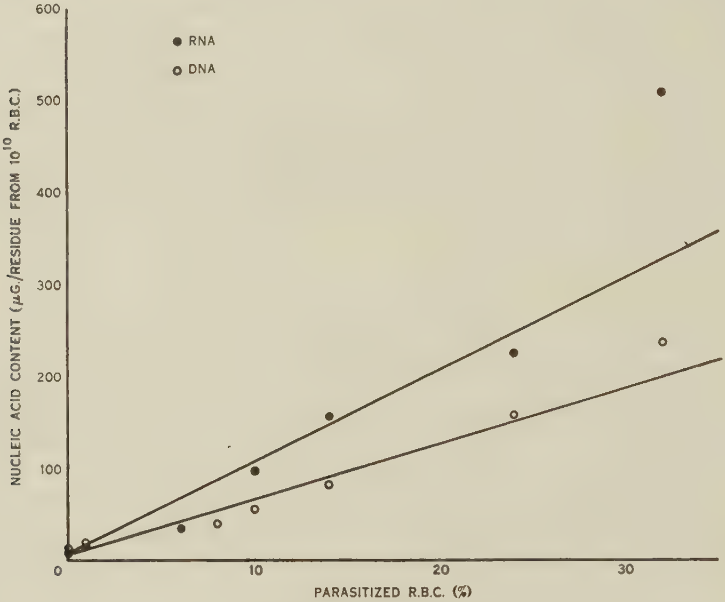

Fig. 1.—Increase in nucleic acid content of blood of mice infected with *P. berghei*.

Table 3

DESOXYRIBOSENUCLEIC ACID CONTENT OF RESIDUE FROM PARASITIZED BLOOD

Parasitized R.B.C. (%)	R.B.C. ($\times 10^{-9}$/ml.)	Leucocytes ($\times 10^{-6}$/ml.)	Total DNA Content (μg./ml.)	DNA Content* of Leucocytes (μg./ml.)	DNA Content† of Parasites and Ghost R.B.C. (μg./ml.)	DNA Content of Residue from 10^{10} R.B.C. (μg.)
0	10·10	6·58	49·9	36·4	13·5	13·4
1	7·76	10·00	70·0	55·2	14·8	19·1
8	5·47	16·50	112·8	91·1	21·7	39·7
10	4·74	9·54	78·0	52·7	25·3	53·3
14	3·17	17·50	112·0	96·6	25·4	80·4
24	3·47	14·14	133·0	78·2	54·8	158·0
32	2·17	15·20	135·0	84·0	51·0	235·0

* Calculated from value given in Table 1 for DNA content per leucocyte.

† These values were obtained by subtracting the DNA content of the leucocytes from the total DNA content.

of parasitaemia was 10 per cent. or more, because the error in determining parasite counts and nucleic acid concentrations at lower levels of infection was much greater.

TABLE 4

MEAN RIBOSENUCLEIC ACID CONTENT PER PARASITE

Parasitized R.B.C. (%)	R.B.C. (×10⁻⁹/ml.)	RNA Content of Parasites and Ghost R.B.C. (μg./ml.)	RNA Content* of Ghost R.B.C. (μg./ml.)	RNA Content of Parasites (μg./ml.)	Mean RNA Content per Parasite (μg.×10⁻⁷)
10	4·74	46·0	4·7	41·3	0·87
14	3·17	48·6	3·1	45·5	1·03
24	3·47	78·9	3·4	75·5	0·91
32	2·17	109·7	2·1	107·6	1·55

* Calculated from RNA content of residue from 10^{10} non-parasitized R.B.C., Table 2.

The mean RNA content per parasite was 1.09×10^{-7} μg. and the mean DNA content per parasite was 0.54×10^{-7} μg. The variation in the results was considerable and the reasons for this are discussed in a later section of the paper.

TABLE 5

MEAN DESOXYRIBOSENUCLEIC ACID CONTENT PER PARASITE

Parasitized R.B.C. (%)	R.B.C. (×10⁻⁹/ml.)	DNA Content of Parasites and Ghost R.B.C. (μg./ml.)	DNA Content* of Ghost R.B.C. (μg./ml.)	DNA Content of Parasites (μg./ml.)	Mean DNA Content for Parasite (μg.×10⁻⁷)
10	4·74	25·3	6·3	19·0	0·40
14	3·17	25·4	4·2	21·2	0·48
24	3·47	54·8	4·6	50·2	0·60
32	2·17	51·0	2·9	48·1	0·69

* Calculated from DNA content of residue from 10^{10} non-parasitized R.B.C., Table 3.

(*c*) *Absorption Spectrum of Parasite Nucleic Acids*

A preparation of free parasites was obtained from infected blood by centrifuging the blood at 2000 r.p.m. for 20 min. The plasma and buffy layers were removed and the red blood cells were washed and haemolysed with saponin. The residue was then washed with saline and the ghost red blood cells removed by careful centrifugation. This procedure resulted in a 40 per cent. loss of parasites and was unsatisfactory for the preceding quantitative experiments.

A sodium chloride extract of the isolated parasites was prepared and the nucleic acids were precipitated as the lanthanum salts (Davidson and Waymouth 1944). Further purification was carried out by decomposition of the precipitate with sodium carbonate and reprecipitation with lanthanum acetate. Finally, a solution of the nucleic acids in sodium carbonate was examined in the Beckman spectrophotometer. The absorption spectrum (Fig. 2) was found to correspond exactly with that of yeast nucleic acid.

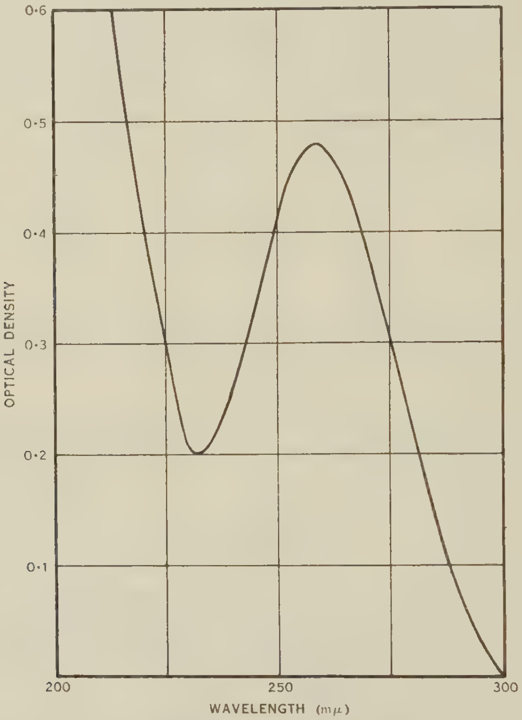

Fig. 2.—Absorption spectrum of nucleic acid extracted from *P. berghei*.

(*d*) *Purine and Pyrimidine Content of Parasite Nucleic Acids*

The procedure of Marshak (1951) was followed for the preparation of RNA and DNA from malaria parasites and of DNA from mouse leucocytes. Isolated parasites (prepared as described in (*c*)) or leucocytes were extracted three times with ice-cold 10 per cent. trichloracetic acid and then with the following fat solvents: acetone-alcohol (twice), chloroform (once), and alcohol-ether (thrice). This exhaustive procedure was necessary to ensure complete removal of all lipids, otherwise hydrolysis of the nucleic acids with perchloric acid would have been incomplete. The residue was digested overnight in 1N potassium hydroxide at 37°C. and the DNA was precipitated with a hydrochloric-trichloracetic acid mixture. The DNA was further purified by resolution in alkali and by reprecipitation and was finally dried over phosphorus pentoxide. The solution which contained the ribonucleotides was kept in a boiling water-bath for 30 min. to destroy the trichloracetic acid, then treated with perchloric acid to remove potassium, evaporated to dryness in a stream of air at 78°C., and dried over phosphorus pentoxide.

The ribonucleotides and DNA were hydrolysed in 72 per cent. perchloric acid at 100°C. for 2 hr. and the hyrolysates chromatographed by the ascending method in 65 per cent. *iso*propanol-2N hydrochloric acid (Wyatt 1951*b*) on Whatman No. 1 filter paper. The purine and pyrimidine spots on the chromatogram were detected by ultraviolet photography (Markham and Smith 1949) and were then cut out and eluted overnight in 4.0 ml. 0.1N hydrochloric acid at 37°C. The densities of the eluates were read at their respective absorption peaks against their corresponding blanks.

TABLE 6

PURINE AND PYRIMIDINE COMPOSITION OF RIBOSENUCLEIC ACIDS

Nucleic Acid	Molar Proportions*				Purines/Pyrimidines (Molar proportions)
	Adenine	Guanine	Cytosine	Uracil	
Yeast (commercial)	1·10	1·20	0·81	0·89	1·35
P. berghei	1·22	1·14	0·62	1·02	1·40

* Calculated to an arbitrary total of 4.00.

The results are given in Tables 6 and 7, together with the corresponding figures for yeast RNA, thymus DNA, and DNA isolated from mouse leucocytes. To permit comparison with similar results published by other authors, the molar proportions of the bases have been calculated to an arbitrary total of 4.00.

TABLE 7

PURINE AND PYRIMIDINE COMPOSITION OF DESOXYRIBOSENUCLEIC ACIDS

Nucleic Acid	Molar Proportions*				Purines/Pyrimidines (Molar proportions)
	Adenine	Guanine	Cytosine	Thymine	
Thymus (commercial)	1·15	0·96	0·80	1·09	1·12
Mouse leucocytes	1·22	0·94	0·82	1·02	1·17
P. berghei	1·21	0·83	0·80	1·16	1·04

* Calculated to an arbitrary total of 4.00.

Whereas the composition of yeast RNA corresponded with other published results (Marshak 1951; Smith and Markham 1950), that of thymus DNA differed considerably from the values given by Marshak (1951) or Wyatt (1951*b*).

Parasite nucleic acids were found to contain the same bases as yeast and thymus nucleic acids. No 5-methyl cytosine was detected by the above method, but unless recourse is taken to the extensive procedure worked out by Wyatt (1951*a*), the possibility of its occurrence in parasite DNA cannot be excluded.

Parasite RNA had a much lower cytosine content than yeast RNA and parasite DNA could be distinguished from both thymus DNA and mouse leucocyte DNA by its lower guanine and higher thymine content. Leucocyte DNA itself had a higher adenine and a lower thymine content than thymus DNA.

IV. Discussion

The values obtained in the present investigation for the DNA content of leucocytes were approximately 20 per cent. lower than those reported by Mandel, Métais, and Cuny (1950). The fact that counting of the leucocytes preceded their treatment with saponin and washing may have resulted in some loss of leucocytes and it is probable, therefore, that the results of Mandel, Métais, and Cuny are the more correct. Nevertheless, as the same procedure was used in the determination of nucleic acids in the residue from infected blood, this error does not affect the results which demonstrate the presence of RNA and DNA in the malaria parasite and an increase in nucleic acid content with an increasing degree of parasitaemia.

For several reasons, the values calculated for the RNA and DNA content per parasite can only be regarded as approximations. The exact number of parasites in the residue was not known, because only the number of parasitized red blood cells was counted; free parasites and the multiple infection of some red cells were ignored. Also, as the counting was carried out before haemolysis of the blood cells, the loss of parasites during this procedure, and during the subsequent washing, could not be estimated. Furthermore, in any one sample of infected blood there were parasites at different stages of the asexual cycle, and in all probability the concentration of RNA and DNA differs at each stage (Deane 1945). Admittedly, as the samples of blood were always collected at the same hour of the day, the majority of the parasites should have been at either the late segmenting or early trophozoite stage, but the proportion in each stage might vary, as might also the number of parasites in other stages. All these considerations are reflected in the variations in the values obtained (see Tables 4 and 5), and these variations are of such an order that the final results cannot be regarded as absolute.

One interesting observation which arose from these calculations was that the RNA content was double the DNA content; Moses (1950) showed that the concentration of RNA in the nuclei of another protozoon, *Paramecium caudatum,* was greater than that of DNA.

The proportions of purines and pyrimidines in the parasite's nucleic acids in no way supported the classical tetranucleotide hypothesis. The composition of parasite RNA found in these experiments was very similar to that found by Markham and Smith (1950) in tobacco mosaic virus. Both are characterized by a low cytosine and a high adenine content. So far, there has been no attempt to classify RNA's according to their base composition.

This is not the case, however, with DNA. Certain regularities in the purine and pyrimidine composition of DNA's have led to the theory that there are two main groups of DNA's. In one group, designated the 'AT type,' the concentration of adenine and thymine exceeds that of guanine and cytosine, whereas in the other group, the 'GC type,' the reverse is the case. Chargaff (1951) has suggested that DNA's of animal origin are of the AT type and those of bacterial origin of the GC type. This classification has been extended by Smith and Wyatt (1951) to other bacteria, bacteriophages, and viruses. Whereas phages T2 and T5 of *Escherichia coli* have the AT type, *E. coli* itself does not belong definitely to one group or the other. Human and bovine strains of *Mycobacterium tuberculosis* and gipsy-moth (*Lymantria* spp.) polyhedral virus have the GC type.

According to this system of classification the malaria parasite, *P. berghei*, has the AT type of DNA, but no significance can be attached to this grouping until further supporting evidence is provided. It would be interesting to examine the composition of other protozoal DNA's in this respect.

V. Acknowledgments

The author wishes to thank Professor W. P. Rogers, Zoology School, University of Adelaide, formerly of McMaster Laboratory, C.S.I.R.O., under whose guidance this work was carried out. Nitrogen determinations were kindly carried out by Dr. K. W. Zimmerman, Division of Industrial Chemistry, C.S.I.R.O., Melbourne.

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A PRELIMINARY STUDY OF THE GENETICS OF DDT RESISTANCE IN HOUSEFLIES

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Summary

A detailed study is reported of the knockdown responses of a highly resistant "Illinois" strain of houseflies and of a non-resistant "Canberra" strain to topical applications of DDT and to exposure to a residual film of DDT. The dosage response curve for the Illinois strain is highly asymmetric, no increase in mortality being observed above a dose of 4 μg. DDT per fly. It is suggested that the resistant population is genetically heterogeneous, being composed of "weak" and "strong" individuals with respect to resistance to DDT.

Eight crosses between Canberra and "weak" Illinois flies were analysed using knockdown response to a dose of 8 μg. DDT per fly as a criterion of resistance. Although the pattern of response was highly variable there was little difference between F_1 and F_2 generations, the F_2 being only slightly more variable than the F_1. No flies in the F_2 fell into the "strong" category.

Five crosses between Canberra and "strong" Illinois flies were analysed similarly. There was a clear-cut segregation in the F_1 and F_2 generations into "strong" and "weak" individuals. The segregation suggests that high resistance in the Illinois strain is controlled by a dominant gene superimposed on the partially resistant constitution of the "weak" type.

The development of DDT resistance in a number of laboratory and field resistant strains of house flies is reviewed briefly and an attempt made to interpret these in the light of the present experiments.

I. Introduction

Since the observation by Sacca (1947) that field populations of the housefly, *Musca domestica* L., had become resistant to DDT and the subsequent demonstration by Lindquist and Wilson (1948) that a DDT-resistant strain could be selected in the laboratory, a large number of papers have appeared on the resistance of field and laboratory populations of houseflies to DDT and other insecticides. Recently two groups of workers have discussed the possible genetic mechanisms which control the resistance of houseflies to DDT. Bruce and Decker (1950*a*, 1950*b*) studied the resistance of offspring resulting from crosses between a field resistant (DDT 111) and a susceptible laboratory strain. The F_1, F_2, and F_{15} generations are reported to have LD50 values intermediate between those of the two parental strains and reciprocal crosses gave identical results. Bruce and Decker suggested that resistance might be described as a multiple gene character which causes indifferent physiological and, perhaps,

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morphological changes. Using a different criterion for the assessment of resistance, Harrison (1951) concluded that DDT resistance is controlled by a single pair of allelomorphs. Individual flies from a resistant strain of Italian origin (Torre in Pietra) were crossed with susceptible flies, the progeny being tested by exposure to a DDT residue in glass cylinders. Knock-down time of each individual fly was noted and the population divided into resistant and susceptible flies on this basis. Results indicated that the F_1 had a resistance similar to, though not identical with, that of the non-resistant strain. In the F_2 generation 75 per cent. of the flies resembled the non-resistant strain.

The discrepant results in these two investigations were obtained on different strains of flies, and using different methods of assessing resistance; further, the published figures do not enable one to assess the importance of all the factors involved in such crosses. In an attempt to clarify the effect of some of these factors we have carried out a more intensive study of crosses between a highly resistant and a non-resistant strain of houseflies.

II. Materials and Methods

The resistant strain used in these experiments was obtained from the Multi-X stock (Bruce and Decker 1951) and was kindly supplied to us by Dr. Bruce of the Illinois Natural History Survey. Stock cultures of these flies have been maintained in our laboratories since October 1951 without further exposure to DDT. Non-resistant flies were derived from a laboratory strain maintained since 1940 by the Division of Entomology, C.S.I.R.O., Canberra.

Flies were reared at 28°C. with constant illumination. Larvae were bred in a slightly modified Peet-Grady medium (West 1951) and the adults allowed to emerge into wire frame cages covered with cloth and plastic. The adults were fed on a mixture of 50 per cent. milk and 5 per cent. sucrose soaked into cotton wool. Handling of the flies was facilitated by immobilizing them with CO_2.

Single-pair crosses were carried out in 4-oz. glass jars and virgin females were ensured by separating pupae into individual specimen tubes and sorting the emerging flies into appropriate pairs.

In all cases the testing procedure followed was to sort the pupae into cages and test at a standard age of 5-8 days after emergence. The DDT used was a commercial sample of 80 per cent. purity. Testing was carried out either by topical applications to the dorsal surface of the thorax of 0.002 ml. of DDT in acetone delivered from an Agla micro-injection syringe or by exposure to a DDT residue of approximately 10 mg./sq. ft. in 1000-ml. beakers. After testing by topical application flies were transferred for observation to clean bottles containing food. The same conditions of temperature and light used for breeding were maintained throughout. When knockdown time was being recorded the following criterion was used:

Flies were considered to be knocked down only when they showed a permanent loss of equilibrium. Frequently, this occurs a considerable time after the flies are first knocked down (Yeager and Munson 1949).

Table 1

NO. OF CANBERRA FLIES DEAD 24 HR. AFTER RECEIVING A MEASURED DOSE OF DDT

DDT (µg. per fly)	0·032		0·048		0·064		0·08		0·096		0·112		0·128		0·16		0·192	
	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead
Male	40	5	80	23	60	48	60	49	61	59	80	79	81	80	60	60	40	40
Female	40	5	80	2	60	3	63	9	60	11	78	38	80	42	60	50	40	37
Total	80	10	160	25	120	51	123	58	121	70	158	117	161	122	120	110	80	77

Table 2

NO. OF ILLINOIS FLIES DEAD 24 HR. AFTER RECEIVING A MEASURED DOSE OF DDT

DDT (µg. per fly)	0·16		0·4		0·8		1·2		1·6		4		8		16		32		64		96	
	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead	No. Used	No. Dead
Male ..	42	5	41	16	43	20	41	21	40	21	39	17	37	20	40	16	44	24	39	24	29	11
Female ..	41	3	40	0	41	11	41	16	40	13	41	28	40	16	41	17	41	22	40	19	36	20
Total	83	8	81	16	84	31	82	37	80	34	80	45	77	36	81	33	85	46	79	43	65	31

III. Results

(a) *Dosage-mortality Curves*

In order to study the variability of response to applications of DDT in the two strains of flies at our disposal, initial dosage-mortality tests were carried out. The results of these tests are given in Tables 1 and 2. The log dosage-probit mortality curves for the two strains are plotted in Figure 1.

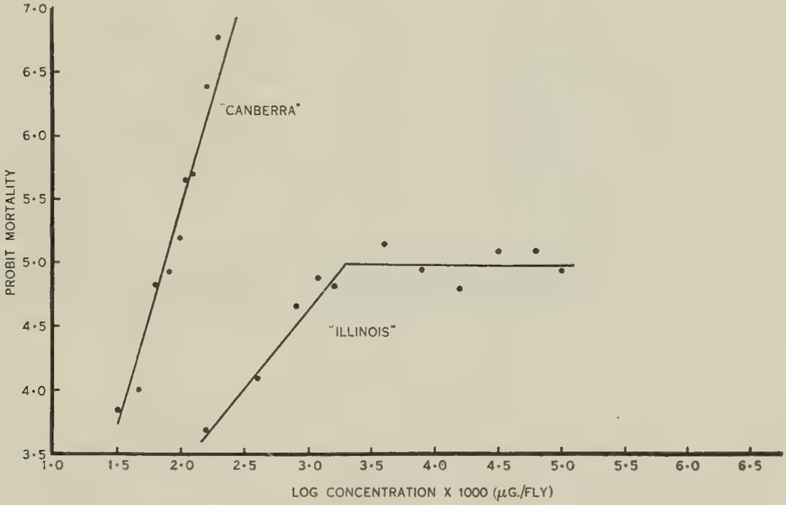

Fig. 1.—Dosage-mortality curves for two strains of houseflies.

While the Canberra flies gave a dosage-mortality response similar to other strains of DDT-susceptible flies, the resistant Illinois flies gave a linear log dosage-probit response relationship up to a concentration of DDT of 4 μg. per fly. At this level approximately 50 per cent. of the flies were alive after 24 hr. and continued to live normally after that. Further increases in the concentration of DDT up to a maximum of 96 μg. per fly resulted in no further increase in mortality.

Any explanation of the results of crosses between Illinois and Canberra flies must take into account the apparent lack of homogeneity in the Illinois population. A method of testing individual flies was developed, therefore, which would enable some discrimination to be made between flies resembling the Canberra stock in their resistance, and those resembling either "weak" Illinois flies, i.e. those unable to withstand a single dose of 4 μg. per fly, or "strong" Illinois flies, i.e. those able to survive a dose between 4 and 96 μg. per fly. It was found that a dose of 8 μg. DDT per fly gave the most clear-cut difference between these three possible types if knockdown time of the flies was recorded. Table 3 and Figure 2 present the knockdown times for Canberra and Illinois stocks.

The Canberra flies show a very rapid knockdown response; 50 per cent. of flies being knocked down after 1 hr., 100 per cent. after 2 hr. Knockdown response of the Illinois flies overlaps slightly with that for the Canberra stock, for although no flies are knocked down in 1 hr., 10 per cent. come down in the second hour. The number of flies knocked down then rises steadily up to 6

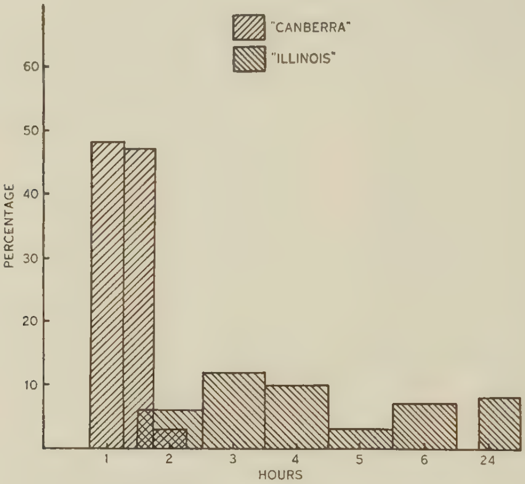

Fig. 2.—Frequency histograms for knockdown by DDT (8 µg. per fly).

TABLE 3

KNOCKDOWN TIMES FOR FLIES RECEIVING A TOPICAL APPLICATION OF 8 μG. DDT IN 0.002 ML. ACETONE

		No. Knocked Down After									
Canberra Strain	No. Used	30 Min.	45 Min.	60 Min.	90 Min.	120 Min.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	24 Hr.
Male	17	10	10	10	17	17	A further 80 flies were tested and observed only after 120 min. They gave 100 per cent. knockdown				
Female ..	14	—	—	5	13	14					
Total	31	10	10	15	30	31					
Illinois Strain											
Male	37	—	—	—	—	3	9	17	18	18	20
Female ..	40	—	—	—	—	2	5	5	6	13	16
Total	77	—	—	—	—	5	14	22	24	31	36*

* A further 82 flies were tested and observed only after 120 min. They gave 14 per cent. knockdown.

hr., when 40 per cent. of the flies are involved. Subsequently little further increase occurs, 53 per cent. of flies remaining alive at the end of 24 hr.

A somewhat similar knockdown response is obtained if the flies are exposed to a DDT residue of 10 mg./sq. ft. in 1000-ml. beakers (not more than 10 flies in each beaker) (Table 4). Canberra flies are all knocked down at 180 min., only 14 per cent. of Illinois flies being knocked down in the same period of time. The knockdown of Illinois flies increases to 49 per cent. at the end of 7½ hr., and remains at this level up to 10 hr. At this stage there is still no mortality among the control flies, but thereafter mortality rises in both control and experimental series. This is due, almost certainly, to starvation, since the flies in these tests, unlike those in the individual dosage response tests, were not fed during the 24-hr. test period.

The knockdown pattern, both in response to individual doses and to exposure to a DDT residue, is in agreement with the information from the dosage-mortality curves. Canberra flies are not only killed by a lower dose of DDT than are the Illinois flies, but at a single dose level corresponding to a point on the "plateau" of the Illinois dose response curve, are knocked down very much more quickly. The knockdown pattern for Illinois flies suggests, as does the dosage-mortality curve, that this stock is heterogeneous for factors influencing resistance, approximately 50 per cent. of the stock having resistance characteristics at a "high" level.

(*b*) *Crosses between Canberra and "Weak" Illinois Flies*

A large number of reciprocal crosses between single Canberra and Illinois flies were set up and the flies allowed to lay their eggs. The eggs were removed and the parents tested for resistance with a single topical application of 8 μg. DDT per fly. Illinois parents that survived less than 6 hr. were classed as "strong." None of the parents was knocked down between 6 and 24 hr.

Eight crosses between "weak" Illinois and Canberra flies were successfully carried through to an F_2 generation, and tests completed on samples of the F_1 and F_2 populations. The results are recorded in Table 5.

One point emerges clearly as a result of these crosses, in contrast to those in which a strong Illinois parent is involved. In the F_1 generations no flies survived more than 6 hr., but in the F_2 generations a small proportion (1.8 per cent.) survived more than 6 hr., but none of these six flies lived for 24 hr.

A more detailed examination of the data in Table 5 reveals that the knockdown pattern for F_1 and F_2 flies is in no case identical with either that for Canberra flies alone or for "weak" Illinois flies alone. Approximately 80 per cent. of the F_1 and F_2 flies were knocked down at the end of 2 hr. and a further 15 per cent. in the succeeding 2 hr. But the figures reveal considerable variation from cross to cross for the knockdown pattern in the first and second hour, both among F_1 and F_2 flies.

This variability in response over a time period critical for discriminating typically "Canberra" knockdown responses from "Illinois" responses suggests that no simple genetic factor is involved whose effects are clear-cut. It seems likely that both genetic and environmental factors contribute to the observed differences in knockdown pattern of F_1 and F_2 flies during the first and second hours. Until further work enables an assessment to be made of the relative

TABLE 4

KNOCKDOWN TIMES FOR FLIES EXPOSED IN 1000-ML. BEAKERS TO A DDT RESIDUE OF 10 MG./SQ. FT.

Canberra Strain	No. Used	No. Knocked Down After																
		60 Min.	75 Min.	90 Min.	120 Min.	150 Min.	180 Min.	210 Min.	240 Min.	270 Min.	300 Min.	330 Min.	360 Min.	390 Min.	420 Min.	450 Min.	600 Min.	24 Hr.
Male	31	13	25	27	—	30	31*	A further 120 flies were tested and observed only at 150 min. They gave 100 per cent. knockdown.										
Female	22	5	8	15	—	17	22											
Total	53	18	33	42	—	47	53											
Knockdown (%)		34	62	79	—	88	100											
Illinois Strain																		
Male	22	—	—	2	3	5	8	10	12	12	12	12	12	12	12	12	12	12*
Female	35	—	—	—	—	—	—	—	—	1	5	6	10	12	15	16	17	24
Total	57	—	—	2	3	5	8	10	12	13	17	18	22	24	27	28	29	36
Knockdown (%)		—	—	4	5	9	14	18	21	23	30	32	39	42	47	49	51	63

* Control flies in 1000-ml. beakers gave nil mortality after 600 min.

importance of these genetic and environmental factors little significance should be attached to the calculation of variance for the F_1 and F_2 populations.

(*c*) *Crosses between Canberra and "Strong" Illinois Flies*

The picture emerging from crosses between Canberra flies and Illinois flies which survive an application of 8μg. per fly is entirely different from that for the "weak" crosses discussed above.

Five such crosses were carried through to completion, and the results of mortality tests carried out on samples of the F_1 and F_2 generations are given in Table 6. Each fly was given a dose of DDT of 8μg. and the mortality after 24 hr. recorded.

The results fall into two distinct groups on the basis of the reaction of the F_1 generation. Crosses 9 and 10 show no mortality among the F_1 offspring, whereas crosses 11, 12, and 13 show more non-resistant than resistant flies in the F_1, the mortality at the end of 24 hr. being 64 per cent. A χ^2 test applied to the data in Table 6 reveals that crosses 9 and 10 are significantly different from 11, 12, and 13. Furthermore, crosses 11, 12, and 13 also differ significantly from one another in the F_2 generation. The difference between these two groups at first suggests that a single dominant gene exercises a major influence in determining survival to the test dose applied. If this is the case the Illinois parents entering crosses 9 and 10 would be homozygous, while those entering crosses 11, 12, and 13 would be heterozygous for this gene. On this basis we should expect 75 per cent. of the F_2 population in crosses 9 and 10 to be resistant individuals. On the basis of random mating among the observed population in the F_1 of crosses 11, 12, and 13, we should expect 33 per cent. resistant individuals in the F_2 generations. It will be seen that the fit to this hypothesis is reasonably good for crosses 9 and 10. But it must be noted that, in the three crosses 11, 12, and 13 where resistant and non-resistant flies are found together in the F_1 and F_2 generation, the number of non-resistant flies is above expectation in every case. This deviation is significant.

It is possible to explain the discrepancy if the dominant gene involved in these crosses also has the effect of reducing the fertility of the flies which carry it. This may indeed be the case. We have made a number of attempts to establish cultures of Illinois flies from crosses between pairs of "strong" flies. Such crosses have always yielded few adults in the first generation, and none of these stocks has been successfully maintained for more than two or three generations.

Other factors, however, are almost certainly involved. One can be illustrated from cross No. 13. The knockdown pattern for the Illinois parent was one displayed by a small percentage of flies in this stock. After receiving the standard dose of 8μg. DDT the fly was recorded in the knockdown stage after 3 hr. At 6 hr. the fly had recovered, and at 24 hr. was still alive. This "strong" fly gave the most discrepant results on the single dominant gene hypothesis for high resistance. But an examination of the knockdown pattern for flies in the F_1 and F_2 generation from this cross (Table 7) shows that a small number of

TABLE 5

KNOCKDOWN FREQUENCIES. FLIES FROM CANBERRA × "WEAK" ILLINOIS CROSSES

Each fly received a topical application of 8 µg. DDT

Cross No.	Illinois Parent	Time of Knockdown of Illinois Parent (hr.)	No. Tested		1 Hr.		2 Hr.		3 Hr.		4 Hr.		5 Hr.		6 Hr.		6-24 Hr.	
			♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
F_1																		
1	♀	2-3	20	23	13	6	7	10	—	3	—	4	—	—	—	—	—	—
2	♀	3-4	21	20	6	6	14	12	1	2	—	—	—	—	—	—	—	—
3	♂	1-2	20	20	—	—	14	6	6	2	—	7	—	2	—	3	—	—
4	♂	2-3	15	15	—	—	15	15	—	—	—	—	—	—	—	—	—	—
5	♀	5-6	19	21	14	10	5	4	—	3	—	1	—	3	—	—	—	—
6	♀	5-6	23	20	9	5	14	4	—	11	—	—	—	—	—	—	—	—
7	♀	5-6	21	18	9	5	12	8	—	—	—	5	—	—	—	—	—	—
8	♀	5-6	41	43	28	17	13	9	—	11	—	6	—	—	—	—	—	—
Total F_1			180	180	79	49	94	68	7	32	—	23	—	5	—	3	—	—
F_2																		
1	♀	2-3	21	21	5	2	16	2	—	6	—	1	—	4	—	1	—	5
2	♀	3-4	20	21	7	2	4	5	9	6	—	6	—	1	—	1	—	—
3	♂	1-2	20	20	6	1	9	5	5	7	—	—	—	3	—	3	—	1
4	♂	2-3	20	20	15	18	3	2	1	—	—	—	1	—	—	—	—	—
5	♀	5-6	20	20	—	—	20	16	—	2	—	2	—	—	—	—	—	—
6	♀	5-6	20	21	—	—	20	20	—	1	—	—	—	—	—	—	—	—
7	♀	5-6	41	40	—	—	41	33	—	7	—	—	—	—	—	—	—	—
8	♀	5-6	21	20	—	—	21	16	—	2	—	2	—	—	—	—	—	—
Total F_2			183	183	33	23	134	99	15	31	—	11	1	8	—	5	—	6

flies in both generations manifest the same phenomenon as the Illinois parent, i.e. recovery after an initial knockdown. None of the flies in any of the other 12 crosses reported here showed this behaviour.

It seems likely that the Illinois parent in cross 13 was one which, though perhaps carrying the "strong" resistant gene, carried other factors which led to early knockdown. The fly would fall into an intermediate category between "weak" and "strong," and in consequence might be expected to yield a larger number of non-resistant flies among its offspring than flies which fall clearly into the "strong" category.

The knockdown pattern for the susceptible flies in crosses 9-13 (Table 7) resembles that for flies in crosses 1-8 (Table 5). Again there is considerable variation in the percentage knockdown in the first and second hour, but in no case is the pattern typical of either Canberra or Illinois flies but lies between these two extremes. This suggests that, whatever the nature of the factors determining the high resistance manifest by the "strong" Illinois flies, these factors are superimposed on a genetic constitution similar to that of the flies placed in the "weak" category.

TABLE 6

NO. OF FLIES ALIVE AND DEAD 24 HR. AFTER RECEIVING A DOSE OF 8 G. PER FLY DDT. CANBERRA × "STRONG" ILLINOIS CROSSES

Cross No.	F_1			F_2		
	No. Used	No. Dead	No. Alive	No. Used	No. Dead	No. Alive
9	38	0	38	39	11	28
10	41	0	41	42	13	29
11	40	24	16	244	162	82
12	24	13	11	165	131	34
13	30	23	7	83	70	13

IV. DISCUSSION

A consideration of the effect on DDT resistance of the crosses recorded above suggests that at least two types of genetic constitution are found among individuals in the resistant population. One type, when crossed with a non-resistant individual, gives rise to offspring with an intermediate resistance and these in turn produce an F_2 generation little different from the F_1. The resistance of such flies seems to be multifactorial. Flies of this type succumb to dosages varying over a range from 0.1 to 4.0 μg. DDT per fly. Part of this variation is due to differences in body weight, for which no allowance has been made in these experiments. Part may be due to uncontrolled environmental factors, of which food may be one of some importance. But it would seem plausible that some at least of the variation is due to different combinations of a group of genes controlling physiological or morphological characteristics of value in resisting the action of DDT.

TABLE 7

KNOCKDOWN FREQUENCIES. FLIES FROM CANBERRA × "STRONG" ILLINOIS CROSSES

Each fly received a topical application of 8 μg. DDT

Cross No.	Illinois Parent	No. Tested		No. Knocked Down After													
				1 Hr.		2 Hr.		3 Hr.		4 Hr.		5 Hr.		6 Hr.		24 Hr.	
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
F_1																	
9	♂	20	18	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	♀	21	20	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	♀	18	22	7	—	1	9	—	4	—	2	—	—	—	—	1	—
12	♀	13	11	—	—	7	4	—	2	—	—	—	—	—	—	—	—
13*	♀	15	15	—	—	13	11	2	2	—	—	(7)	—	—	—	—	2
F_2																	
9	♂	22	20	6	2	—	2	1	2	—	—	—	—	—	—	—	—
10	♀	19	20	2	—	3	1	1	3	—	1	—	—	—	—	—	—
11	♀	123	121	69	54	8	10	6	10	—	1	—	1	—	1	—	2
12	♀	86	79	—	—	66	52	3	4	—	—	1	3	—	—	2	—
13*	♀	40	43	31	38	2	—	(2)	1	—	(3)	2	—	—	(2)	1	2

* The Illinois parent in cross No. 13 was knocked down at 3 hr. but subsequently recovered and was alive at the end of 24 hr. Several flies in the F_1 and F_2 from this cross exhibited the same phenomenon. The figures for recovery are given in parentheses.

The second type of fly in the resistant population has a genetic constitution similar to that of flies of type 1 but possessing in addition a genetic factor which enables the individual to survive single applications of DDT from 4 μg. up to at least 96 μg. These flies, when crossed with non-resistant individuals, show a segregation in the F_2 generation which suggests that the genetic factor involved is a single dominant gene. But there is also some evidence that flies carrying this gene are less fertile than those possessing its allelomorph only.

The occurrence of two distinct genetic types of flies among the resistant strain analysed here emphasizes the danger inherent in basing discussions of resistance on LD50 values. Complete information on the dosage response pattern of the population studied should be supplied if a picture of the mechanism by which resistance is built up in field and laboratory strains is to be obtained. On the basis of what little information is available an attempt can be made to outline a general mechanism for the acquisition of resistance in strains of *Musca domestica.* Two intensively studied laboratory strains have been selected from non-resistant stocks by exposure over successive generations to DDT. The Orlando strain used in studies by Lindquist and Wilson (1948), Wilson and Gahan (1948), King and Gahan (1949), King (1950), Pratt and Babers (1950), and Lindquist *et al.* (1951) increased in resistance only slightly up to the 22nd generation. However, between the 22nd and 55th generation a 50-fold increase in resistance occurred, and the population was much more variable than before. The resistance does not seem to have increased much since the 55th generation, but King and Gahan (1949) reported that some flies in the population could not be killed by very high concentrations of DDT. King (1950) reported a decline in resistance of the Orlando strain when selection pressure was removed at the 53rd generation, and Lindquist *et al.* (1951) also demonstrated a decline in resistance at the 85-88th generation following removal of selection pressure at the 75th generation.

The DDT 1 strain selected by Bruce and Decker (1950*a*) from an NAIDM laboratory strain showed a somewhat similar development. There was little increase in resistance up to the twelfth generation but after that a very rapid increase was recorded for the next five generations. Bruce and Decker (1950*b*) did not observe any decline in resistance over 34 generations when the selection pressure was removed.

Two partially resistant field strains DDT 111 (Bruce and Decker 1950*a*) and Torre in Pietra (Harrison 1952) have also been subjected to selection for DDT resistance in the laboratory. Both these strains showed only the rapid phase of development of resistance. In the Torre in Pietra strain selection for eight generations yielded a population with characteristics similar to those reported here for the strain derived from Multi-X, i.e. a highly asymmetric dosage-mortality curve. Roma, another Italian strain partly resistant to DDT, has been subjected to selection by Harrison under conditions identical with those for Torre in Pietra. After eight generations no significant increase in resistance took place.

Further studies on selection in the laboratory of partially resistant field strains have been reported from California. Perry and Hoskins (1950) were able to increase the resistance of the Laton strain five times at the LD50 value by only one generation of selection, and this Super Laton stock showed an increased variance. A Bellflower field strain from California had LD50 values higher than either Laton or Super Laton, and was more variable than either of these two strains.

This analysis indicates that most field populations of houseflies must be polymorphic with respect to factors controlling resistance to an insecticide like DDT and the evolution of a DDT-resistant strain must follow a pattern rather like the following:

The first stage is a relatively slow process involving the accumulation of a number of independent factors each of which, singly or in combination, confers resistance on the fly. Following this slow increase is a rapid rise in resistance of the population, owing to selection of a highly resistant phenotype controlled probably by a single pair of alleles. This highly resistant population can never be rendered homogeneous because of the low fertility associated with the high resistance factor. It seems likely that the high resistance factor cannot be selected initially because the selective advantage this factor tends to confer is less than its selective disadvantage owing to the lower fertility associated with it.

V. ACKNOWLEDGMENTS

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STUDIES ON THE DIGESTION OF WOOL BY INSECTS

IX. SOME FEATURES OF DIGESTION IN CHEWING LICE (MALLOPHAGA) FROM BIRD AND MAMMALIAN HOSTS

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Summary

Some aspects of digestion in eight species of Mallophaga infesting birds and mammals have been examined.

The anatomy and histology of the alimentary canal are described and the relatively poor tracheation of the crop and midgut recorded.

The bird-infesting species all regularly ingest feather particles and the poultry body louse in addition obtains fresh blood from its host. In these species the food in the crop is subjected to the action of digestive juices passed forwards at regular intervals from the midgut and, as a consequence, the pH (about 8.0) and oxidation-reduction potential (about —200 mV.) of both of these regions is the same. The presence of a comb of teeth at the posterior end of the crop in Amblycera and the arrangement of the inlet and outlet of the crop in Ischnocera prevent the passage of most intact feather particles into the midgut, which receives partially digested material with disorganized structure. Free sulphydryl groups can be detected in the crop and midgut, and the ingestion of cobalt-impregnated feathers results in the formation of a characteristic brown, cobaltic-cysteine complex. Metallic sulphides are not produced, indicating that, as in dermestid larvae, the cystine of feather keratin is reduced to cysteine, but that further breakdown to hydrogen sulphide (which is characteristic of the clothes moth larva, *Tineola*) does not occur.

Neither hair nor wool could be detected regularly in the digestive tract of the mammal-infesting species examined, which instead appear to ingest mainly epithelial debris and skin secretions. In the sheep louse *Damalinia ovis*, the slightly acid (pH 6.5), oxidizing (greater than + 77 mV.) midgut digestive juices are not passed regularly into the crop. Free sulphydryl groups could not be detected and it is doubtful if wool would be digested during passage through the alimentary canal.

I. INTRODUCTION

It is now well established that larvae of the clothes moth *Tineola* and of at least three species of carpet beetle are able to digest wool keratin, and something is known about the mechanism of this process (Waterhouse 1952*a*, 1952*b*, 1952*c*). Although there is good circumstantial evidence that the chewing lice of birds are able to digest feather keratin (Eichler 1937), additional proof is necessary. Furthermore, it is not known whether the chewing lice of mammals can or do digest the hair (or wool) of their host, although it is often stated in

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textbooks that species infesting sheep and goats cause damage by partially cutting and weakening the wool or hair fibres.

The evidence that bird Mallophaga digest feather keratin is firstly that the feather barbs and barbules are consumed, secondly that there are partly digested feathers in the crop and midgut where sulphydryl groups can also be demonstrated and conditions are at least mildly reducing (Duspiva 1936), and thirdly that some species can be reared for a period *in vitro* on host feathers (Barber 1921; Martin 1934; Wilson 1934, 1939). It is clear, however, that this diet is not pure feather keratin and that the feathers must have been contaminated with various accessory growth factors unless, perhaps, these were supplied by contaminating microorganisms or by symbionts. It is well known, for example, that many Mallophaga harbour rickettsiae, although their relation to the metabolism of the lice is not known. It is also well known that Mallophaga have a varied diet when living on their avian hosts. For example, Waterson (1926) records that the protecting sheaths of growing feathers, feather fibre, down, skin scurf, scabs, blood, their own egg shells and cast skins, and perhaps other Mallophaga as well are ingested. An examination of the crop contents further indicated the presence of small particles of various minerals and other fortuitous feather contaminants. There is no doubt that blood regularly forms portion of the diet of some species (Strindberg 1917; Kotlan 1923; Wilson 1933; Eichler 1937; Crutchfield and Hixson 1942; Bouvier 1945), but, for others, blood alone is not an adequate diet. Thus, although the poultry body louse (*Eomenacanthus stramineus*) is recorded as actively feeding on the blood of its host (Wilson 1933), the poultry head louse (*Lipeurus heterographus*) will ingest only dried blood and then lives only 3 days on this diet alone, whereas it thrives on dried blood plus feathers. Silk and blood is an inadequate diet for this species (Wilson 1934). Further, the slight pigeon louse (*Columbicola columbae*) will not feed on a blood clot, although it can be induced to feed on blood-stained feathers (Martin 1934).

Very little is known about the digestion of those Mallophaga which live on mammals. The cattle biting louse (*Damalinia bovis*) can be reared *in vitro* on fresh cattle hair sprinkled with dried brewer's yeast (Matthysse 1944) and the biting louse of sheep (*Damalinia ovis*) on greasy wool, skin scurf, and powdered yeast (Scott 1952), but it is not known whether these species digest the keratin components of these diets.

It will be shown in this paper that the three species of bird Mallophaga examined regularly digest feather keratin, and some features of this process are described. The biting louse of sheep, however, seldom if ever ingests wool and probably cannot digest fibres which may be fortuitously ingested.

II. Methods

The following species of Mallophaga were examined:

Sub-order Amblycera

Family Menoponidae	*Eomenacanthus stramineus* (Nitzsch), the poultry body louse.

Boopidae	*Heterodoxus* sp. (from the red wallaby, *Macropus ruficollis*).
Gyropidae	*Gyropus ovalis* Nitzsch, the oval guinea pig louse.
Sub-order Ischnocera	
Philopteridae	*Columbicola columbae* (L.), the slight pigeon louse.
Trichodectidae	*Lipeurus caponis* (L.), the poultry wing louse. *Damalinia ovis* (L.), the sheep body louse. *D. bovis* (L.), the cattle biting louse. *D. caprae* (Gurlt), the goat body louse.

It can be seen that the species are representative of both bird- and mammal-infesting families of each of the two sub-orders of Mallophaga.

All species (except *Heterodoxus* and *Gyropus*) were collected from their respective hosts as required and maintained in an incubator at 36-37°C. and 74 per cent. relative humidity, physical conditions roughly similar to those employed by the authors, cited above, who bred lice *in vitro*. Only a small number of *Heterodoxus* (about 50) were available. This species died in a few hours at 36°C., although it lived for several days at 18°C. Similarly, *Gyropus* lived far better off its host at 18-20°C. than at 36°C.

In most experiments indicators or metal salts were added to the diets fed, which consisted of freshly obtained white breast or neck feathers, unwashed hair, or greasy, scurfy wool from the respective hosts. The addition of materials to these diets was carried out by dipping the source of keratin in 70 per cent. alcohol (to ensure wetting), rinsing in distilled water, immersing in the test solution, and drying. No attempt was made to remove contaminating debris from the keratin before treatment. On most of the diets lice survived the duration of a test (1-2 days) and often for a longer period, particularly the ischnoceran species. It was not possible to induce *Heterodoxus* to feed on any of the diets offered and *Gyropus* did not ingest the treated diets at all readily unless the chemicals were introduced into the hair of the living guinea pig and the lice allowed to feed *in situ*.

Because of their relatively transparent cuticle it was generally possible to select lice for dissection which had fed on the treated diet and also to follow certain of the colour changes of indicators in living lice. It was also possible to observe peristalsis and antiperistalsis in the crop and midgut, particularly if the lice were examined with dark-field illumination.

III. Results

(*a*) *Morphology of the Digestive System*

(i) *Alimentary Canal.*—The following are the more important papers which contain information on the general morphology of the digestive system of Mallophaga, although most of the species used in the present investigations have apparently never been adequately dealt with: Nitzsch 1818; Kramer 1869;

Grosse 1885; Snodgrass 1899; Cummins 1913; Strindberg 1916; Ewing 1924; Blagoveshchensky 1931; Risler 1951; Haug 1952.

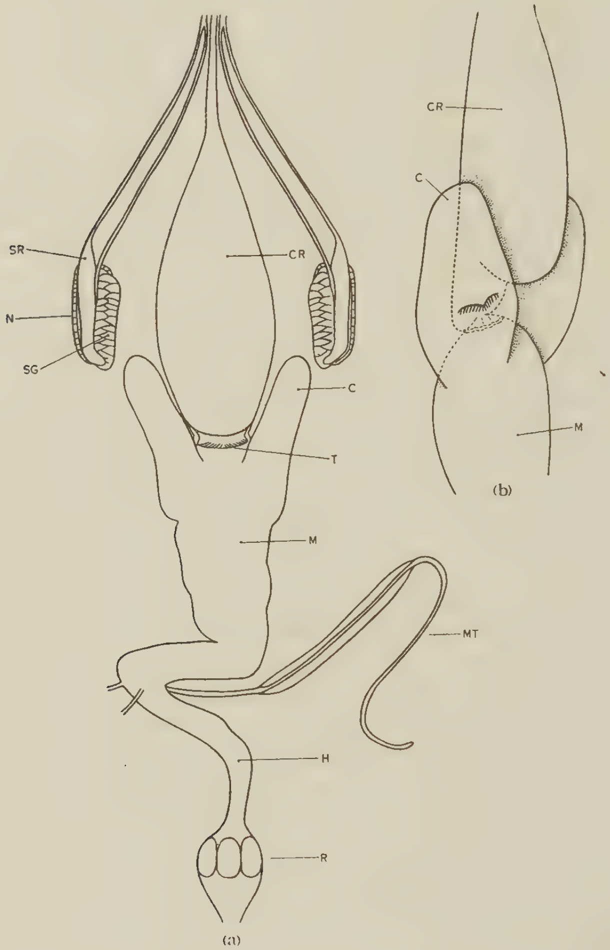
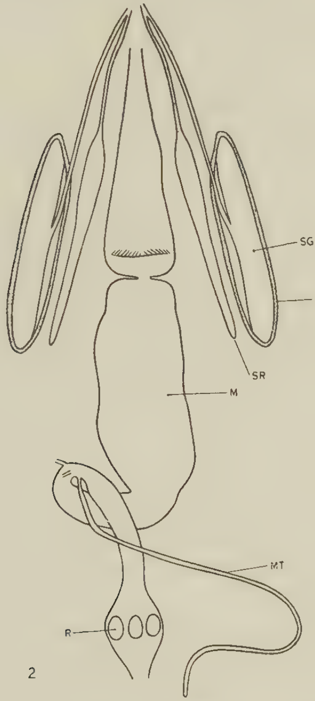


Fig. 1.—(*a*) Alimentary canal of *E. stramineus*, dorsal view.
(*b*) Diagram showing relationship of crop teeth and oesophageal invagination.
C, caeca; *CR*, crop; *H*, hindgut; *M*, midgut; *MT*, malpighian tubules; *N*, perioesophageal nephrocytes; *R*, rectal pads; *SG*, salivary gland; *SR*, salivary reservoir; *T*, comb of teeth.

The general morphology of the alimentary canal of Mallophaga conforms to three basic patterns (Snodgrass 1899). In the sub-order Amblycera the oesophagus expands into a crop before leading directly into the midgut (Figs. 1, 2, and 3). On the other hand in most families of Ischnocera the oesophagus leads into a large, expanded crop diverticulum interrupting the direct passage of food to the midgut. This diverticulum extends anteriorly only for a short distance, but posteriorly almost to the end of the body (Fig. 4). Nearly opposite the entrance of the oesophagus into the crop the foregut leads out of the crop and, soon after, into the midgut. The ischnoceran family Trichodectidae is an exception to this arrangement, for here the crop is a simple diverticulum which leads off by means of a constricted neck from the lower end of the oesophagus, the latter continuing on directly to the midgut (Fig. 5).



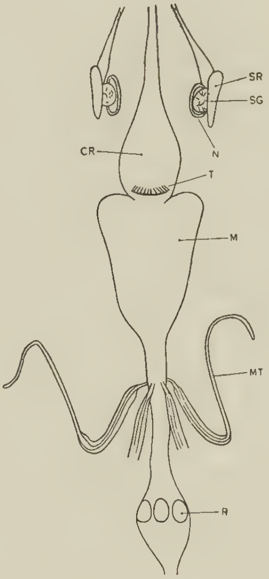
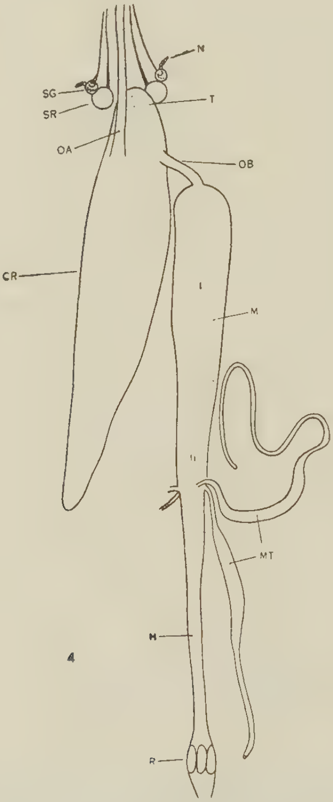


Fig. 2.—Alimentary canal of *Heterodoxus* sp.

Fig. 3.—Alimentary canal of *G. ovalis*. Details as in Figure 1.

Except in the Trichodectidae the crop always possesses teeth of characteristic shape, grouping, and restricted distribution (Cummins 1913; Waterson 1926). In the Amblycera the teeth are situated in a circular or semicircular fashion near the posterior end of the crop. Thus in *Eomenacanthus* (Plate 1, Fig. 1) the teeth are arranged in two contiguous arcs arising in the dorsal wall of the crop and projecting ventrally. Each tooth is C-shaped. The free anterior arm of the C is long, slightly curved, and may reach 50 μ in length, whereas the posterior arm is short (up to about 10 μ in length). The long anterior arms each have a very fine tip. These crop teeth form an effective comb-like sieve which prevents any large particles of food from entering the midgut. A somewhat similar arrangement of teeth occurs in *Heterodoxus* and *Gyropus*. In *Eomenacanthus* the poorly developed oesophageal invagination (see also *Heterodoxus*, Plate 2, Fig. 2) leads out slightly in front of the blind posterior end of the crop and is situated where its anterior end receives maximum protection from the comb of teeth. It scarcely protrudes at all into the midgut, having the form of a short, thin-walled, funnel-shaped structure (Fig. 1*b*). In the Ischnocera the teeth occur singly or in small rows in the anterior diverticulum of the crop and mainly in the dorsal wall. In *C. columbae* and *L. caponis* (Fig. 4; Plate 1, Fig. 2) the groups may contain up to six teeth, and in *L. caponis* (considerably the larger of these two species), they may reach a length of about 7 μ. In the Ischnocera, as in the Amblycera, the oesophageal invagination is poorly developed.



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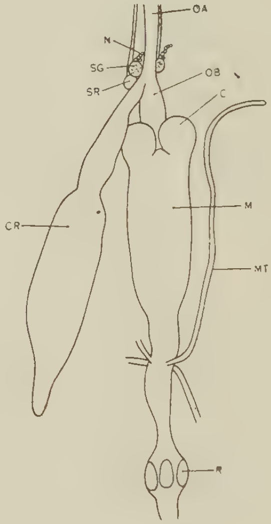

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Fig. 4.—Alimentary canal of *C. columbae*, with crop rotated and moved to the left.

Fig. 5.—Alimentary canal of *D. ovis*.
C, caeca; *CR*, crop; *H*, hindgut; *M*, midgut; *MT*, malpighian tubules; *N*, perioesophageal nephrocytes; *OA*, oesophagus region *A*; *OB*, oesophagus region *B*; *R*, rectal pads; *SG*, salivary gland; *SR*, salivary reservoir; *T*, position of teeth.

The mallophagan midgut is simple and is either fairly straight (*C. columbae, D. ovis*) or possesses a single bend or loop at its posterior end (*E. stramineus, Heterodoxus*). There are sometimes two anterior caeca (*E. stramineus, D. ovis*), but these may be absent (*Heterodoxus, C. columbae*). The anterior two-thirds or three-quarters of the midgut has a greater diameter than the posterior region and may be differentiated histologically (see later).

There are always four malpighian tubules (one pair often longer than the other), which generally have in their basal half a differentiated zone of greater opacity than the rest. This zone, which is generally of greater diameter also, may be very restricted or it may occupy almost half of the tubule. In some species (e.g. *Heterodoxus, E. stramineus*) portion of the tubule may be pigmented.

The hindgut narrows shortly after the entrance of the tubules, but expands again into a conspicuous rectum supplied with six rectal pads.

(ii) *Salivary Glands.*—Lying on either side of the crop in the vicinity of the anterior end of the midgut there are two salivary organs which probably represent a relatively opaque gland and a more transparent reservoir (Figs. 1-5). In the Amblycera the gland, and more particularly the reservoir, are relatively elongate, whereas in the Ischnocera both gland and reservoir are more or less spherical (Snodgrass 1899; Stowe 1943; Risler 1951; Haug 1952). The ducts often arise near, or at, the anterior ends of the salivary organs (*C. columbae, D. ovis*) but, in some species (e.g. *E. stramineus, Heterodoxus*) the duct of the salivary gland (but not that of the reservoir) arises near the centre of the gland. The ducts of gland and reservoir unite in the head and the resultant common ducts of each side fuse shortly before discharging into the salivarium.

(iii) *Perioesophageal Nephrocytes.*—Associated with each pair of salivary organs in Ischnocera is a group of some 7-24 spherical or polygonal cells (the number varies with the species), which were regarded by Snodgrass (1899) as constituting glands whose ducts discharged into the anterior end of the crop. Kramer (1869) observed these cells, which are generally binucleate and, although he was unable to see any efferent duct, he regarded them as bearing some relation to salivary secretion because of their position. Grosse (1885), however, discounted their salivary function, since he found similar cells associated with the fat body in other parts of the body. Nuttall and Keilin (1921) examined similar cells in the sucking louse *Pediculus* (Order Anoplura) and demonstrated that they were nephrocytes. They suggested that Snodgrass may have taken for a "duct" leading from the nephrocytes either tracheae, nerves, muscle fibres, or real salivary ducts with which these particular nephrocytes are intimately associated.

These two groups of cells (each about a dozen in number) are clearly visible in *C. columbae* and *D. ovis* (Figs. 4, 5). Injection into the haemocoele of a saturated solution of ammonia carmine in saline demonstrated clearly that they were indeed nephrocytes, since they (and small groups of cells distributed throughout the fat body) readily took up this dye.

Associated with the salivary glands in the Amblycera (*E. stramineus, Heterodoxus,* and *Gyropus*) there is a garland of cells forming an ellipse and lying close to the surface of the salivary organs (Figs. 1, 2, 3). The affinity of these cells in *E. stramineus* for injected ammonia carmine indicates that they too are nephrocytes and it appears that they are the counterparts of the salivary or perioesophageal nephrocytes of the Ischnocera.

(*b*) *Histology of the Digestive System*

(i) *Alimentary Canal.*—There is little published information on the histology of the alimentary canal, although some regions have been incidentally figured (but with little detail) by authors such as Snodgrass (1899) and Strindberg (1916). A more detailed account of the midgut histology of *Bovicola* (= *Damalinia*) *caprae* and *E. stramineus* is about to appear (Haug 1952).

There appear to be no unusual features in the histology of the foregut (except the presence of teeth, mentioned previously) and there is no indication of any secretory function of the epithelium even in species (see later) in which food breakdown proceeds rapidly in the crop. The crop is very well supplied with muscles, especially circular muscles, and the latter are a particularly noticeable feature of the philopterid crop.

The bulk of the midgut epithelium is composed of mature cells with some immature cells and many small regenerative cells (Plate 1, Figs. 3, 4, 7; Plate 2, Figs. 1, 3). The mature cells usually have a well-defined striated border, and are at times completely separated from the basement membrane by regenerative cells. Their nuclei (each with a fuchsinophilic nucleolus) therefore tend to lie towards the gut lumen. On the other hand, the immature cells (and their nuclei) lie closer to the basement membrane and expose far less striated border to the lumen (Plate 1, Fig. 3). Their cytoplasm is denser than that of the mature cells. Haug (1952) interprets what are here regarded as mature and immature cells as two distinct cell types, each producing a characteristic secretion. The regenerative nuclei are very numerous and are slightly smaller than those of the mature cells. In most species they form an almost continuous layer adjoining the basement membrane. Although the difference is not always marked, the epithelium of the anterior two-thirds of the midgut is generally higher (Plate 1, Fig. 5) and has the histological appearance of greater activity than the relatively lower epithelium of the posterior third (Plate 1, Fig. 6). The midgut epithelium is generally higher and more "active" in appearance in *E. stramineus* (Plate 1, Figs. 5-7) than in the other species examined. However, this may have been due to greater difficulties in obtaining satisfactorily fixed preparations of this species, since poor fixation was found to produce more vacuolated, elongated, and "active" cells than good fixation. Granules which stain heavily with Bodian's protargol method occur in some individuals (especially *Heterodoxus*, Plate 1, Fig. 8) near the lumen of the mature cells. No structure resembling a peritrophic membrane of the type arising at the anterior end of the midgut was seen. However, in several species (e.g. *E. stramineus*) the food in the posterior half of the midgut was loosely compacted into a cylindrical shape. A membrane envelops these food masses and can be shown by van Wisseling's test to contain chitin. In *Lipeurus* the cylinder of food material is regularly of smaller diameter than the lumen and first becomes visible about one-third of the way down the midgut. There is no doubt that a peritrophic membrane is produced by the midgut epithelium in some species.

There are few unusual features in the hindgut. The anterior quarter often possesses a higher or more convoluted epithelium than the remainder (e.g. *Lipeurus,* Plate 2, Fig. 4) but the six well-developed rectal pads, each with a number of centrally placed nuclei, are the most conspicuous feature (Plate 2, Figs. 5, 7).

(ii) *Salivary Glands.*—The epithelium of the salivary glands is high and fuchsinophilic, the nuclei large and oval, and there are frequently granules which stain with Bodian (Plate 2, Figs. 8, 9). The saliva is discharged into a relatively small central lumen. By contrast the epithelium of the reservoir is flat, the nuclei elongate, and the central lumen very large (Plate 2, Fig. 8). Histological details for other species are given by Strindberg (1916), Stowe (1943), and Risler (1951).

(iii) *Perioesophageal Nephrocytes.*—These have a structure typical of other nephrocytes. The cytoplasm is not very dense, nor is it differentiated and there are frequently two nuclei per cell (Plate 2, Fig. 6). They have been figured by Risler (1951) for *D. caprae.*

(*c*) *Tracheation of the Alimentary Canal*

Harrison (1915) published the only detailed study of the respiratory system of Mallophaga, but paid little attention to the tracheation of the alimentary canal, which he reported very briefly as being well developed. This is incorrect as far as the crop and midgut are concerned in any of the species used in the present study.

There is no important difference in the degree of tracheation of the alimentary canal in *Eomenacanthus, Columbicola, Lipeurus,* or *Damalinia.* In each the crop does not appear to be tracheated, except occasionally and very sparsely at its posterior end in *Eomenacanthus.* The midgut is almost devoid of tracheae except for the anterior quarter, which is poorly supplied with a few small tracheae. Even these tracheae branch into relatively few tracheoles which supply air directly to only a small proportion of epithelial cells. In *Eomenacanthus,* tracheae are generally restricted to the region of the caeca. The anterior quarter of the hindgut is well tracheated and the rectal pads are richly supplied. The salivary glands are also supplied with several tracheal branches.

As in other species, the crop of *Heterodoxus* and *Gyropus* is poorly tracheated. However, the anterior midgut is supplied with more tracheae than observed in other Mallophaga examined, although the tracheoles reach a far smaller proportion of the epithelium than observed in many other insects (Day 1951). The posterior portion of the midgut and the hindgut are tracheated as in the other species.

(*d*) *Movement and Microscopic Examination of Food Undergoing Digestion*

In *Eomenacanthus* the crop is customarily kept full of blood (obtained fresh from the host) and feather particles up to 600-800 μ in length. Under polarized light it is evident, from widely differing degrees of birefringence, that the feather barbs are in various stages of digestion.

The crop contracts at frequent intervals, driving its contents alternately backwards against the comb of teeth at its posterior end and forwards up into the oesophagus almost as far as the head. At the same time the anterior two-thirds of the midgut undergoes waves of contraction and relaxation. During contraction (which is mainly antiperistaltic in nature) its semi-fluid contents are forced up into the tips of the large, sac-like caeca and at times also forwards into the crop, the comb of teeth being simultaneously displaced forwards. Fluid and semi-solid material are then returned to the midgut, most feather particles being strained out in this process by the comb of teeth, although occasionally small particles are not withheld.

In the posterior third of the midgut (which is flexed to the left) there is comparatively little contraction and its contents are in a far less violent state of movement. It will be seen later that the contents of this region are more acid and less reducing than those of the remainder of the midgut.

Stained food was observed to enter the rectum 6 hr. after feeding commenced at 37°C.

In the wallaby louse (*Heterodoxus*), collected from the fur of its host, which had been skinned some hours earlier, the crop, midgut, and rectum had red to dark brown contents and blood proved to be present. In addition there was a good deal of solid irregularly shaped birefringent material in the crop, which appeared to be skin and epithelial debris from the host. There was no evidence that hair had been ingested. The midgut contained much fluid, together with a small number of weakly birefringent solid masses. Both the crop and midgut showed rather regular and vigorous peristaltic and antiperistaltic movements. In these the food was driven back and forth in the crop and, at intervals, fluid and smaller pieces of solid material passed through the crop teeth into the midgut, the larger solids being retained. Shortly afterwards an antiperistaltic wave would return some of the midgut contents to the crop.

Forward movement of contents from the midgut into the crop was not observed in *G. ovalis.* The food of this species consists largely of epithelial debris, skin secretions, and perhaps skin from its host. Neither blood nor hair was ever observed in the digestive tract and no damage could be detected by microscopical examination of host hair from heavily infested areas. This confirms the report of Ewing (1924) for two guinea pig lice (*G. ovalis* and *Gliricola porcelli*). He believed, furthermore, that the cutting and piercing nature of the *Gliricola* hypopharynx indicated that serum was also ingested by this species, although blood was never observed in the digestive tract. He reported that the skin of a guinea pig heavily infested with *G. porcelli* usually did not show any sign of abrasion but that the hair tended to become brittle. He observed that this species thrusts its head into the opening of a hair follicle while holding the hair and believed that the products of the oil glands, which open through the mouths of the hair follicles, probably also constitute an important item of diet.

In the pigeon louse, *C. columbae,* as in the poultry wing louse, *L. caponis,* the food passes rapidly down the oesophagus into the crop, which, when full, extends from the mid line of the mesothorax into the beginning of the fifth abdominal segment, where it lies to the left of the mid line. The most conspicuous elements of the food which packs the crop are the feather barbules, many of which may be a length almost equal to that of the crop, although shorter pieces are also present, particularly when no food has been ingested for some time. In addition to barbules, a small number of hard mineral particles are present and also material which is apparently skin scurf. Blood was not seen in lice obtained directly from the host, although lice kept on feathers on which blood had been dried were found to have ingested it along with feather particles. These observations confirm the report of Waterson (1926) who found small grains of quartz, mica, olivine, felspar, a lepidopterous scale, a seed coat, and fungal spores in the philopterid crop. These feather contaminants were present in addition to various portions of the feather itself and also of skin scurf, scales, blood (when available), cast egg shells, and cast skins of lice.

The oesophagus enters the right side of the crop and leads out at about the same level ventrally (Fig. 4). It is clear, therefore, that the long, slender barbules have little or no chance of passing directly through the crop into the midgut. Furthermore, once the barbules have passed into the crop there is equally little chance of ends being oriented so that long, intact barbules can pass out of the crop towards the midgut. Examination under polarized light indicates that the longer barbules in the crop are generally highly birefringent, whereas the shorter pieces are frequently less birefringent, suggesting that some disorganization of structure has already occurred in the latter.

In living, resting lice the crop undergoes regular antiperistaltic movements of considerable magnitude, each movement arising at the posterior end of the crop and being followed immediately by a peristaltic movement in the opposite direction. Crop movements in Philopteridae have also been recorded by Waterson (1926). The vigorous antiperistaltic movements drive the crop contents against the groups of teeth in the blind anterior end of crop, although these teeth are so small that it is not certain that they play an important part in triturating the food. The vigorous contractions also cause fluid and solid material (which shows little or no birefringence) to pass into the region of the foregut between the crop and the midgut. The contents of this latter region are then, as a general rule, immediately returned to the crop, but at intervals are passed on to the midgut. The anterior region of the midgut is also involved in both anti- and peristaltic movements. In these processes the midgut and hindgut are drawn forwards and then allowed to relax posteriorly by associated contraction and relaxation of muscles at the anterior end of the midgut. In addition to the mass forward and backward movement, the hindgut itself contracts and relaxes, coiling and uncoiling as it does so.

On no occasion was there visible in the *Columbicola* midgut any material which exhibited either the form or the birefringence of even partially degraded feather, although amorphous material and dyes ingested with the feathers were clearly visible therein. This confirms the findings of Waterson (1926), who was unable to detect unaltered feather fibres in the midgut of a philopterid. There are evidently exceptions in the group, however, since small, partially digested feather particles were often visible in the midgut of the poultry wing louse, *L. caponis.* Careful observation demonstrated that, at intervals, some of the contents of the anterior midgut were passed forwards into the crop. This observation explains the data (see later) demonstrating that in *Columbicola* and *Lipeurus,* as in *Eomenacanthus,* the contents of the crop and the anterior region of the midgut have the same pH, oxidation-reduction potential, and reactions to the feeding of metals.

In the sheep body louse, *D. ovis,* the crop leads off dorsally from the oesophagus through a slightly constricted tube and extends nearly to the posterior end of the abdomen, often lying slightly to the right of the mid line. It contains a good deal of poorly birefringent material which is apparently mainly skin or skin debris. There are also occasionally present granules and small, highly birefringent, needle-shaped bodies, the former being mineral in nature. On two occasions only of many individuals examined was wool (a single short length) observed in the digestive tract (midgut) and it therefore appears most unlikely that *D. ovis* ever actively ingests wool fibres. These small pieces of wool showed no signs of digestion. The crop undergoes relatively weak and infrequent anti- and peristaltic movements and, in common with the crop of other members of the Trichodectidae, possesses no teeth. There is a good deal of evidence to indicate that it is mainly a temporary storage organ and it appears that, unlike the philopterid crop, it can be bypassed by food travelling directly down the oesophagus into the midgut. Not infrequently is it empty or almost empty when the midgut contains food after recent feeding. Shortly after feeding on dyed, scurfy wool the midgut was filled with dyed fluid with either very little solid material or with a certain amount of almost non-birefringent, finely divided material of no apparent structure. This further suggests that wool is not readily ingested, for even if pieces were chewed off the sides of fibres they should retain their birefringent properties for some time after ingestion. Stained food passed completely through the alimentary canal in less than 16 hr. at 37°C.

(*e*) *The pH of the Digestive Tract*

The pH records obtained by adding sulphonphthalein indicators to the food of three representative species of Mallophaga are summarized in Table 1.

In *Eomenacanthus* the contents of the crop and the anterior two-thirds of the midgut have the same alkaline pH (8.0-8.4). However, except when food has just passed on, the contents of the posterior midgut are, like the hindgut contents, slightly acid (6.4-7.2).

In *Columbicola* (and in *Lipeurus*) the pH of the contents of the first part of the oesophagus (*OA,* Fig. 4) appeared to be the same as that of the food although this region seldom contained sufficient food for accurate records to be obtained. Although the feathers fed were acid to both phenol red and cresol red, the food in the crop, in the short region of the oesophagus leading into the midgut (*O*), and in the anterior two-thirds of the midgut was alkaline to phenol red and within the colour change range of cresol red, indicating a pH of 7.8-8.0. Bromthymol blue gave slightly lower values (6.8-7.1) than those quoted for crop and midgut, but experience has shown that this indicator is not as reliable in this type of experiment as phenol red or cresol red. The contents of the posterior third of the midgut have a pH of about 6.5 and those of the hindgut about 5.8, although both may occasionally record slightly higher values, presumably due to recent passage of food.

In *D. ovis* the pH of the crop contents was greatly influenced by that of the food. When recently fed on greasy, scurfy wool exhibiting the coloration of either acid or alkaline cresol red, the crop contents were correspondingly coloured. They did, however, tend to be influenced towards a pH of about 6.5, the value of oesophagus region *B* and of the entire midgut.

TABLE 1

pH OF THE CONTENTS OF THE ALIMENTARY TRACT OF THREE SPECIES OF MALLOPHAGA

Species	Foregut			Midgut		Hindgut	
	Oesophagus *A*	Crop	Oesophagus *B*	Anterior	Posterior	Anterior	Rectum
E. stramineus	8·0-8·4			8·0-8·4	6·4-7·2	6·4-7·2	
C. columbae	Same as food	7·8-8·0	7·8-8·0	7·8-8·0	About 6·5	About 5·8	
D. ovis	Same as food	Similar to food (about 6·5)	About 6·5	About 6·5		5·8-6·0	5·0-5·5

(*f*) *Oxidation-reduction Potential of the Digestive Tract*

The oxidation-reduction potential of various regions of the digestive tract (Table 2) was determined by feeding the range of indicators employed in earlier studies (Waterhouse 1952*b*, 1952*c*).

In *Eomenacanthus, Columbicola,* and *Lipeurus* very similar results were obtained. The contents of both crop and anterior two-thirds of the midgut are very reducing, having a potential in the range —170 to —220 mV. (indigo disulphonate was reduced, whereas brilliant alizarine blue remained oxidized). The posterior third of the midgut was coloured a greenish yellow by indigo disulphonate, indicating partial reduction and an Eh of about —100 to —115 mV. In the hindgut indigo tetrasulphonate and gallocyanine exhibited their oxidized colours, indicating an oxidizing potential. Blue inclusions were observed in the epithelium of the anterior portion of the *Columbicola* midgut after feeding on thionine and indigo disulphonate.

In *Damalinia* the picture was quite different. Reducing conditions were not encountered in any region of the gut. Thus, in the crop and midgut the potential was greater than + 77 mV. and in the anterior and posterior regions of the hindgut even more oxidizing. (Thionine was not reduced in any region, an observation supported by feeding a range of indicators which change at more negative potentials.) Furthermore, tetrazolium blue was not reduced during passage through the gut, indicating that reducing conditions were not encountered at any stage (Rutenburg, Gofstein, and Seligman 1950; Waterhouse 1952*c*). Both toluylene blue and methylene blue were absorbed from the digestive tract and appeared in the fat body and other internal organs.

Gyropus was not examined in the same detail as *Damalinia*, but it is relevant to record that indigo tetrasulphonate exhibited its oxidized colour throughout the entire alimentary canal. In this louse the crop and hindgut were acid, and the midgut alkaline, to phenol red.

TABLE 2

OXIDATION-REDUCTION POTENTIAL (mV.) OF THE CONTENTS OF THE ALIMENTARY TRACT OF THREE SPECIES OF MALLOPHAGA

Species	Crop	Midgut		Hindgut	
		Anterior	Posterior	Anterior	Rectum
E. stramineus	−175 to −220	−175 to −220	About −115	>+30	
C. columbae	−170 to −215	−170 to −215	About −100	>+70	
D. ovis	>+77	>+77		>+97	>+124

(g) *Metal Feeding Experiments*

Lice were fed on feathers, wool, or hair which had been immersed in 5 per cent. solutions of nickel or copper sulphates, cobalt, cadmium or ferric chlorides, silver nitrate, lead acetate, or in saturated sodium tellurate (about 4 per cent.).

With none of the species did any of the elements (except cobalt and tellurium) produce any visible colour in the food undergoing digestion or in the faeces, although microchemical tests indicated that treated food had been ingested. It is clear, therefore, that metal sulphides are not formed during digestion.

In *Eomenacanthus*, *Columbicola*, and *Lipeurus* feeding on cobalt-enriched feathers the crop, midgut, and anterior region of the hindgut contained light brown food, whereas the contents of the rectum and faeces were a darker brown. Furthermore, when sodium tellurate was ingested the crop, midgut, and hindgut contained black material. The nitroprusside reaction indicated that free — SH groups were present in the crop and midgut. It is clear, therefore, that the disulphide bonds of feather keratin are reduced with the production of cysteine or cysteine peptides and, in the presence of cobalt, of a brown cobalt-cysteine complex (see Waterhouse 1952*c*).

Application of the ferrocyanide test to *Columbicola* fed on iron-enriched feathers indicated that ferric iron was present throughout the midgut epithelium. In this louse, and more particularly in *Lipeurus,* there was generally a far higher concentration of iron in the posterior midgut than elsewhere. Low concentrations of ferric iron could only be detected in this region in *Lipeurus* obtained directly from its host. In *Eomenacanthus,* whose iron intake is high because of the regular presence of blood in its diet, the entire midgut (and particularly the caeca) stained relatively heavily, but there was no preferential accumulation in the posterior region of the midgut. The salivary glands, perioesophageal nephrocytes, and malpighian tubules also stained lightly.

In *D. ovis* it is clear that the course of digestion is different from that in bird-infesting species. Thus, no deviation from normal coloration occurred when cobalt-impregnated food was ingested and, furthermore, no region of the alimentary canal gave a positive nitroprusside reaction. However, when sodium tellurate was fed the contents of the midgut were blackened, although the colour of the crop contents was unaffected. The faeces contained black material which was visible also in the midgut epithelium. This is an apparently anomalous result, since it was tentatively suggested for dermestid larvae that the dark colour was caused by reduction to metallic tellurium (Waterhouse 1952*c*). Whatever the mechanism of the reaction and the nature of the dark material, it is evident that this reaction cannot be taken to indicate intensely reducing conditions.

After *D. ovis* had fed on iron-enriched food, ferric iron was detected throughout the midgut epithelium and after feeding on barium-enriched food, barium was present throughout the midgut epithelium and in the enlarged regions of the malpighian tubules.

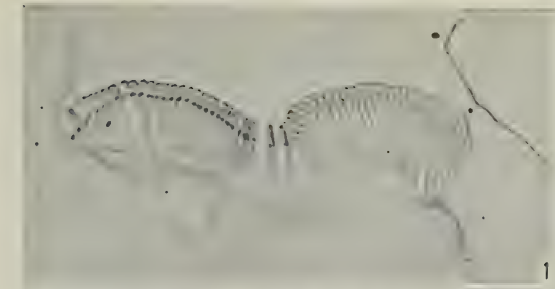
IV. Discussion

In the Mallophaga examined there are no anatomical features of the alimentary canal which have not been broadly covered by previous work (Snodgrass 1899; Cummins 1913). It is perhaps worth emphasizing, however, that the pair of glands described by Snodgrass (1899) as discharging into the crop are, in fact, groups of nephrocytes. The midgut histology is somewhat unusual in that there are numerous regenerative nuclei, which form an almost continuous layer adjoining the basement membrane, but are not aggregated to form nidi. This might be taken to indicate an active, but rather unspecialized, type of epithelium. The tracheal supply of the crop and midgut is poorly developed, as in other insects having the capacity to digest keratin (Day 1951; Waterhouse 1952*c*). It is, however, also poorly developed in mammal-infesting Mallophaga which, if one can generalize from *Damalinia,* do not maintain reducing conditions in their digestive tract. Thus it appears that, whereas effective maintenance of highly reducing conditions may demand poor tracheation, this feature alone will not produce a negative redox potential.

A very striking feature of digestion in the bird-infesting Mallophaga examined is its dynamic nature. The continuous and vigorous circulation of digestive juices from the midgut into the crop and back again, carrying with them partly degraded food, results in the crop being a far more important site for digestion than it is usually held to be in most other insects. The regular flushing of the crop contents with digestive juices would appear to be more important in this process than mechanical breakdown, for the crop teeth in the Amblycera act mainly as a sieve and in the Ischnocera the teeth are small and sparse. The mineral particles in the crop may play some part in mechanical breakdown, even if far less important than those in the crop of their avian host. It is probable that the small mineral grains are ingested as fortuitous feather contaminants from the habit of many birds of taking dust baths. The oesophageal invagination of Mallophaga is poorly developed and funnel-shaped and this region would appear to function mainly as a sphincter during the periods when the contents of the crop and midgut are subjected independently to their respective contractions and relaxations. There is no evidence that the oesophageal invagination functions in aspirating digestive enzymes from the midgut into the crop, as has been suggested for the well-developed invagination of *Periplaneta* (Day and Powning 1949). Indeed, there is no need to postulate such a function in these Mallophaga in view of the vigorous anti-peristaltic waves of the midgut and the shape of the invagination.

Regurgitation of fluid from the midgut to the crop occurs to a very much smaller extent in the mammal-infesting Mallophaga examined. It was observed in *Heterodoxus,* but could not be detected in *Gliricola* or *Damalinia.* The fact that the pH of the contents of the *D. ovis* crop is similar to that of the food ingested and does not rapidly approximate that of the midgut supports the belief that there is little forward passage of fluid from the midgut in this species.

There is clear evidence that bird-infesting Mallophaga are capable of digesting feather keratin by a process similar to that in dermestid larvae (Waterhouse 1952*c*) and that they do not carry the degradation as far as hydrogen sulphide, as in larvae of the clothes moth *Tineola* (Waterhouse 1952*a*). The pH of the crop and midgut is slightly alkaline (about 8.0) and the oxidation-reduction potential very reducing (about — 200 mV.). The cystine of the keratin molecule is reduced to cysteine and the free — SH groups of the latter can be detected. When cobalt salts are added to the feather diet the characteristic brown, soluble, cobaltic-cysteine reaction product is formed in the crop and midgut. Metallic sulphides are not produced. It is interesting to note that feathers have only about two-thirds of the cystine content of wool or hair (Graham, Waitkoff, and Hier 1949), which may render them more readily digested, and that skin has an even lower cystine content (Block 1951). Fresh blood is a normal part of the diet in some species (e.g. *E. stramineus*) whereas others only ingest dried blood when this occurs as a feather contaminant. In relation to their dietary requirements it is of interest that a lower population of *E. stramineus* is carried on poultry suffering from several types of avitaminoses than on well-nourished poultry (Kartman 1949). Absorption evidently occurs throughout the midgut, but evidence from iron absorption suggests that the posterior midgut may be specially concerned in this process. This latter region is less involved than the rest of the midgut in continuous exchange of fluid with the crop.



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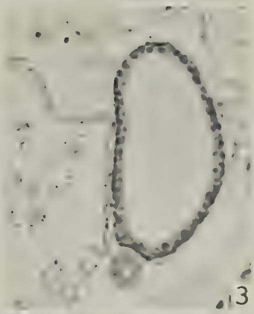

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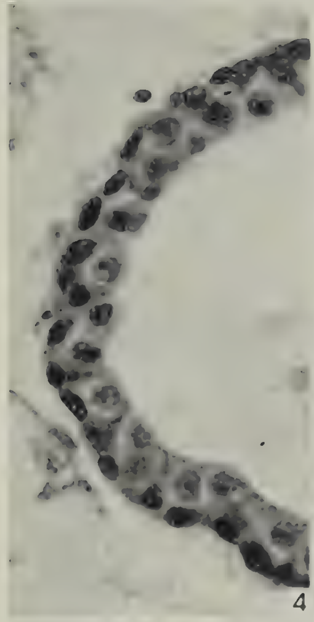

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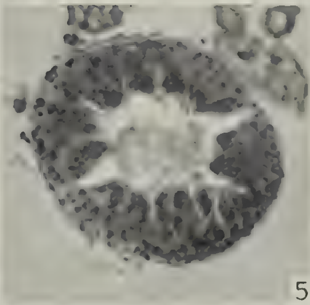

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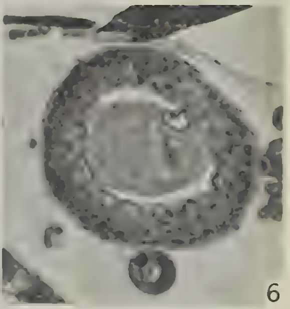

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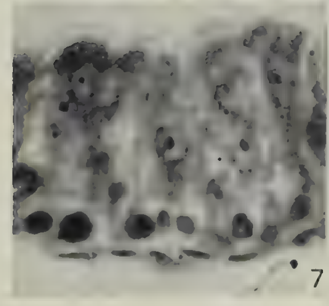

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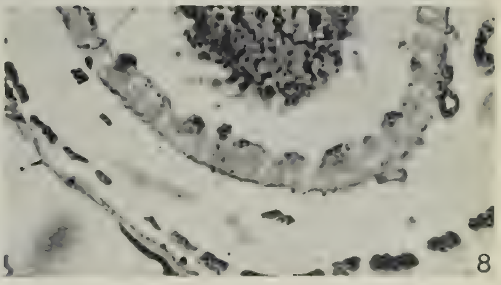

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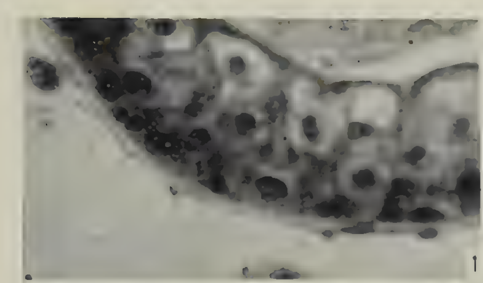
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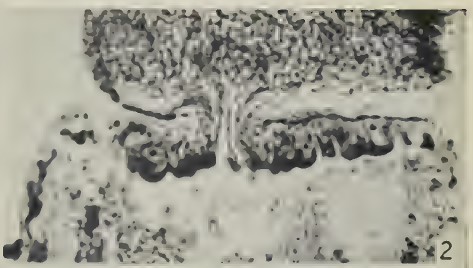
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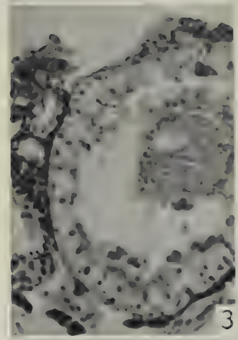
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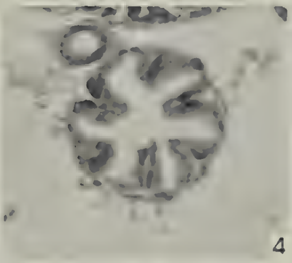
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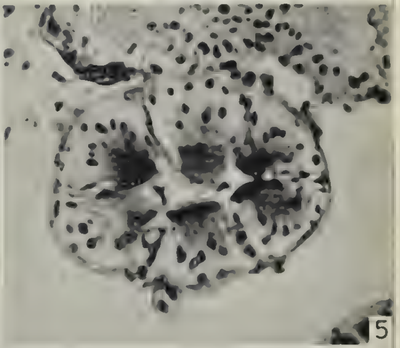
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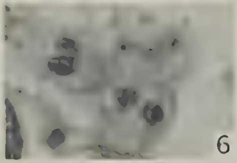
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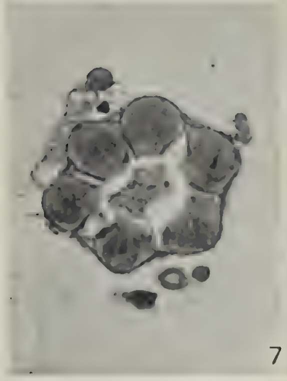
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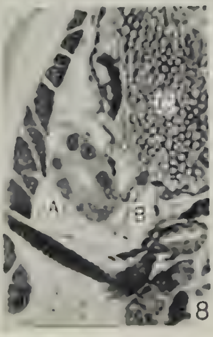

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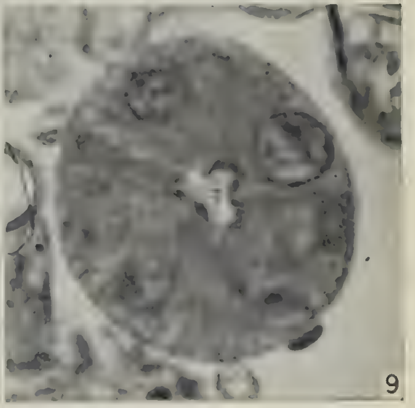
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The mammal-infesting Mallophaga examined do not appear to utilize keratin. In *D. ovis* the midgut pH is slightly acid (about pH 6.5), the oxidation-reduction potential is oxidizing, and no —SH groups can be detected, data which alone would throw doubt upon the ability of this species to digest keratin. In addition, wool is seldom ingested and, when occasionally observed in the digestive tract, is not visibly degraded. Its food appears to consist mainly of skin, skin scurf, wool wax, and any other materials contaminating the fibres, which suggests that its food is similar to that of other mammal-infesting Mallophaga (Eichler 1937). It is clear that the presence of *D. ovis* causes irritation to its host, for heavily infested sheep can often be detected by the rolls of rubbed wool on the surface of the fleece and a general "plucked" appearance. Whether the irritation and the greatly increased scurfiness of the lousy sheep is due merely to the crawling of lice on the tender skin, or whether, as suspected, the lice also chew the surface layer of skin has not been determined. It is relevant to mention, however, that as blood does not form a regular part of their food any attack on the skin must be confined to its outermost layers, which may result in tissue fluids, but not blood, being available also.

It may be unsafe, however, to generalize on the basis of the mammal-infesting species examined that this group is unable to digest keratin. Thus there are reports in textbooks (e.g. Essig 1942) that the goat louse, *Bovicola* (= *Damalinia*) *caprae*, damages the hair of its host, although Haug (personal communication) states that hair particles are only rarely observed in the digestive tract of this species and, when present, are scarcely, if at all, digested. His observations are supported by an examination of about a dozen specimens of *D. caprae* which were dissected several days after removal from their host and left in a tube with goat's hair. No hair was detected in the gut, although some epithelial debris was present. If the reports are correct that hair is both ingested and digested, there must be important differences in the digestive physiology of related species. If some mammal-infesting species do regularly ingest the hair of their host it is possible, from the relative diameters of the hair and the midgut lumen, that attack would take the form of chewing pieces off the side of a hair. Even pieces of this nature should, however, be readily distinguishable by their high degree of birefringence, although small, extensively damaged pieces of hair would almost certainly be more rapidly attacked than lengths of intact hair.

V. Acknowledgments

I am indebted to Mr. N. J. B. Plomley, Medical School, University of Sydney, who identified the wallaby louse as an undescribed species of *Heterodoxus*, and to all those whose assisted in the supply of living lice, especially Miss M. Scott and Mr. D. Murray, McMaster Animal Health Laboratory, C.S.I.R.O., and Mr. L. K. Whitten, Animal Research Division, Department of Agriculture, New Zealand. Thanks are due to Mrs. M. A. Halsey for technical assistance. I also wish to acknowledge the kindness of Dr. G. Haug, Tübingen, for sending the proof of his paper during the final stages of preparation of this paper.

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Explanation of Plates 1 and 2

Plate 1

Fig. 1.—Comb-like crop teeth of *E. stramineus*.

Fig. 2.—Small rows of crop teeth of *L. caponis*.

Fig. 3.—T.S. midgut of *D. ovis* showing low epithelium with many regenerative nuclei. Bodian.

Fig. 4.—T.S. midgut of *D. ovis*. Bodian.

Fig. 5.—T.S. anterior midgut of *E. stramineus*, showing relatively high epithelium. Bodian.

Fig. 6.—T.S. posterior midgut of *E. stramineus*, showing relatively low epithelium. Bodian.

Fig. 7.—Anterior midgut of *E. stramineus*. Bodian.

Fig. 8.—T.S. midgut of *Heterodoxus* showing granular inclusions in cells. Bodian.

Plate 2

Fig. 1.—T.S. midgut of *L. caponis*. Bodian.

Fig. 2.—L.S. crop and midgut of *Heterodoxus* illustrating poorly developed oesophageal invagination. Bodian.

Fig. 3.—T. S. midgut of *Heterodoxus*. Bodian.

Fig. 4.—T. S. anterior hindgut of *L. caponis*. Bodian.

Fig. 5.—T.S. rectal pads of *C. columbae*. Mallory.

Fig. 6.—Perioesophageal nephrocytes of *D. ovis*. Bodian.

Fig. 7.—T.S. rectal pads of *D. ovis*. Bodian.

Fig. 8.—T.S. *E. stramineus*, showing salivary gland (*A*), salivary reservoir (*B*), and crop (*C*) containing blood. Bodian.

Fig. 9.—T.S. salivary gland of *D. ovis*. Bodian.

A CHEMICAL ANALYSIS OF BREWER'S MEDIUM FOR THE AEROBIC CULTURE OF ANAEROBES

By V. B. D. Skerman*

[*Manuscript received October* 20, 1952]

Summary

Polarographic methods for the estimation of thioglycollic acid, cysteine, and glutathione in culture media are described.

A study of the rate of oxidation of thioglycollic acid and cysteine in a peptone-yeast-extract medium in the presence and absence of glucose and agar has shown that neither glucose nor agar (0.1 per cent.) influences the rate of oxidation. The oxidation of 0.1 per cent. thioglycollic acid in a medium 7 cm. deep exposed to air occurs in 80 hr.; 0.1 per cent. cysteine HCl approximately 120 hr.

The disulphides formed on oxidation are partially hydrolysed on heating, the extent depending on the time and temperature. The continued usefulness of media used for the "aerobic" culture of anaerobes appears to be dependent on this property.

I. Introduction

Brewer's medium or one of its modifications has been used extensively for the cultivation of anaerobes in sterility tests on various medicinal substances. In his discussion on the function of his medium Brewer (1940) made the following statement, which largely stimulated the investigation:

"Some effort has been made to find an explanation for the seemingly reversible reaction taking place in the medium, but no definite conclusion has been reached. It has been found that 80 cc. of sterile air per minute can be bubbled through a tube of the medium for thirty minutes, and it will still support growth of *Clostridium novyi,* one of the most strict anaerobes."

The constituents of Brewer's medium which might be expected to achieve and maintain an anaerobic state are glutathione in the pork infusion and the thioglycollic acid, aided perhaps by glucose. Both of these substances contain the autoxidizable sulphydryl group. In the glutathione it is present as the amino acid cysteine. The oxidation of the sulphydryl groups to the disulphides would result in the removal of oxygen from the medium and the reduction of the oxidation reduction potentials to a more negative level. It seems logical to assume that the complete oxidation of the sulphydryl groups would result in the destruction of the anaerobic state and hence the usefulness of the medium. The investigations reported in this paper were primarily conducted to determine the rate of oxidation of these components.

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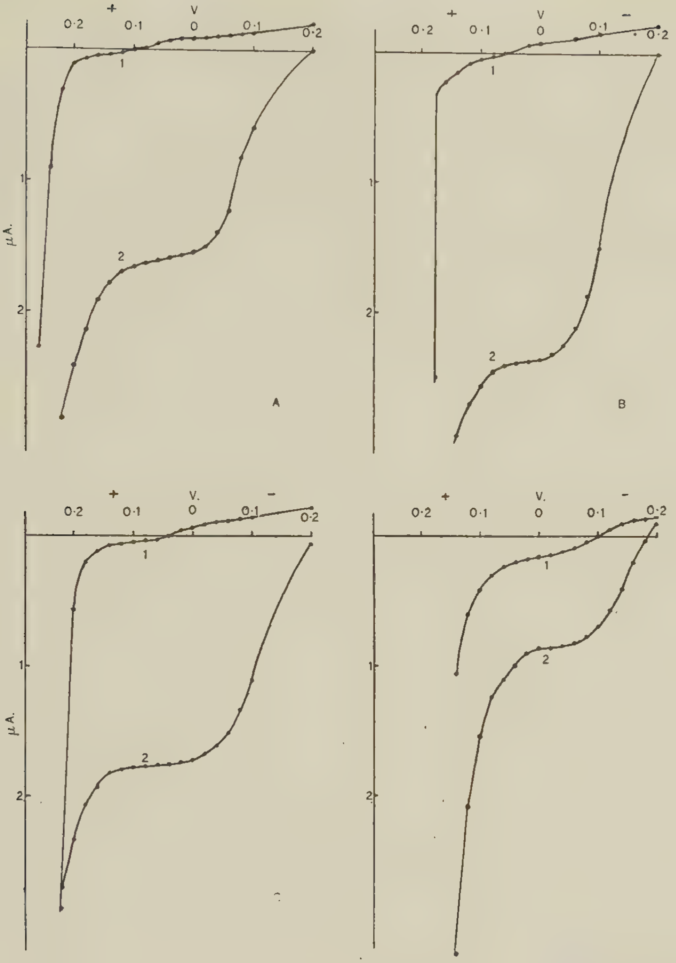

Fig. 1.—*A.* Current-voltage curves for deoxygenated (1) 0.01N HClO$_4$, and (2) 4.3×10^{-4}M thioglycollic acid in 0.01N HClO$_4$, using a saturated calomel reference electrode.

B. Current-voltage curves for deoxygenated (1) 0.01N HClO$_4$ containing 10 per cent. YEPW, and (2) 6.3×10^{-4}M thioglycollic acid in 0.01N HClO$_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode.

C. Current-voltage curves for deoxygenated (1) 0.001N HCl, and (2) 4.5×10^{-4}M thioglycollic acid in 0.001N HCl, using a saturated calomel reference electrode.

D. Current-voltage curves for deoxygenated (1) YEPW at pH 3, and (2) 1.8×10^{-4}M thioglycollic acid in YEPW at pH 3, using a saturated calomel reference electrode.

II. Methods of Analysis

(*a*) *Thioglycollic Acid*

Kolthoff and Barnum (1940) described a method for the estimation of cysteine in 0.1N perchloric acid. The latter provides a supporting electrolyte in which the equilibrium potential of the dropping electrode is sufficiently positive to permit analysis of other substances with a limiting current potential more positive than the reference electrode and it maintains a pH sufficiently low to prevent oxidation of the sulphydryl group during analysis.

The same procedure was examined by the author for the determination of thioglycollic acid. Perchloric acid (0.01N) was substituted for 0.1N perchloric acid to reduce risk of explosion, and a concentration of 36.5 p.p.m. (0.00043M) of the concentrated thioglycollic acid was found to yield, in the absence of oxygen, a well-defined anodic wave with the limiting current measurable at 0.06 V. *v.* S.C.E. (Fig. 1*A*). This represented the analysis of pure thioglycollic acid in perchloric acid. In practice, however, the thioglycollic acid was employed at a concentration of 1000 p.p.m. (0.012M) in a medium consisting of 1 per cent. "Bacto-peptone," 0.5 per cent. "Bacto-Yeast extract," and 0.5 per cent. NaCl. This medium will be referred to in the paper as YEPW. For some purposes 1 per cent. glucose and 0.1 per cent. agar were added.

It was necessary to determine what effect these substances had on the production of the sulphydryl wave. Kolthoff and Barnum (1940) stated that hydrochloric acid was unsatisfactory as a supporting electrolyte for cysteine determinations since the anodic chloride wave interfered with the measurement of the cysteine wave. The YEPW medium contains chloride in *c.* 0.1M concentration. With an initial concentration of 1000 p.p.m. thioglycollic acid a dilution of 1/10 in perchloric acid was necessary to reduce the sulphydryl wave to measurable limits. This dilution contained *c.* 0.01M chloride—sufficient to give an anodic wave of *c.* 80 μA. It was suspected that this might interfere with the direct measurement of thioglycollic acid in YEPW.

The formation of the wave was examined in the following supporting electrolytes:

(i) 0.01N $HClO_4$,

(ii) 0.01N $HClO_4$ containing:
   (1) 10 per cent. YEPW,
   (2) 10 per cent. YEPW containing 0.1 per cent. agar,
   (3) 0.05 per cent. NaCl,
   (4) 0.1 per cent. "Bacto-peptone."

The polarograms for ii (1) are shown in Figure 1*B*. The presence of the YEPW caused a small change in the residual current and produced a steep anodic wave at *c.* 0.17 V. *v.* S.C.E. Comparison with the residual current curves (not illustrated) for ii (3) and ii (4) showed that this wave was due to the chloride. The peptone caused a movement in the wave to a more positive value than that obtained with perchloric acid alone.

The agar had no effect on either the residual current or the sulphydryl wave.

Since the chloride had no apparent effect on the determination of thioglycollic acid in perchloric acid an attempt was made to determine it in hydrochloric acid alone. Figure 1*C* shows the residual current (1) and thioglycollic acid wave (2) obtained in deoxygenated 0.001N HCl. The lower chloride ion concentration is responsible for the more positive potential at which the chloride wave appears as compared with that found in Figure 1*B*. The residual current curve and thioglycollic acid wave were practically identical with those obtained in 0.01N $HClO_4$. There was obviously no chloride interference similar to that reported by Kolthoff and Barnum for cysteine.

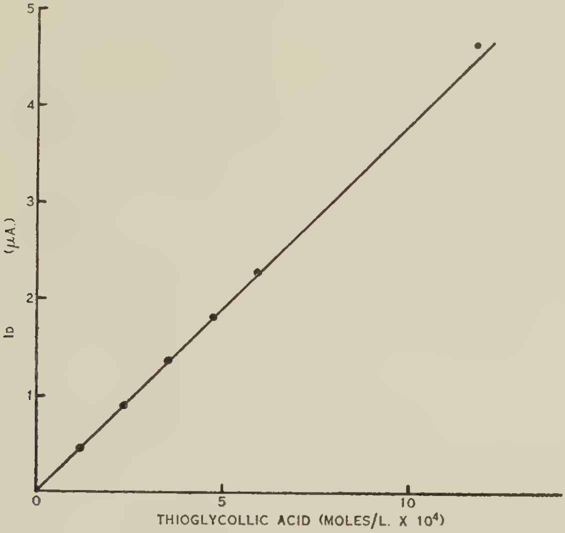

Fig. 2.—Diffusion current-thioglycollic acid calibration curve.

This finding suggested that direct analysis of small concentrations of thioglycollic acid, e.g. 0-50 p.p.m., might be determined directly in YEPW by acidification with hydrochloric acid to pH 3. Figure 1*D* shows the residual current curve (1) and sulphydryl wave (2) obtained in this manner. The curves, though similar in other respects to the previous waves, were displaced to a more negative potential with the limiting current measurable at —0.02 V. *v.* S.C.E.

The presence of 0.1 per cent. agar had no effect on these curves.

Relationship between the diffusion current and thioglycollic acid concentration.—For the purpose of the investigation the maximum amount of thioglycollic acid present in a 1/10 dilution of YEPW was 100 p.p.m. (0.0012M). The diffusion current was found to be proportional to the concentration of thioglycollic acid to within 0.5 per cent. over the range of 0-100 p.p.m. (see Fig. 2). The ratio was found to be the same in all the supporting electrolytes tested both in the presence and absence of agar.

Oxygen had to be excluded from all tests since it interferes with the measurement of the sulphydryl wave. Its removal was effected by bubbling commercial nitrogen through the sample till a zero oxygen reading was obtained at — 0.6 V. *v.* S.C.E. (Skerman and Millis 1949).

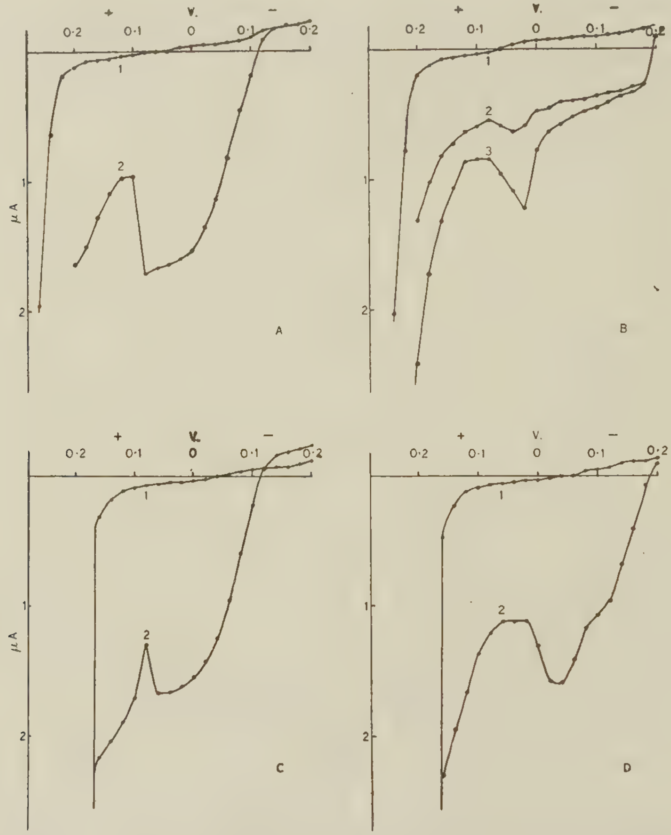

Fig. 3.—*A.* Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$, and (2) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$, using a saturated calomel reference electrode.

B. Current-voltage curves for deoxygenated (1) 0.001N HCl, (2) 6.4×10^{-4}M cysteine HCl in 0.001N HCl, and (3) 12.8×10^{-4}M cysteine HCl in 0.001N HCl, using a saturated calomel reference electrode.

C. Current-voltage curves for deoxygenated (1) 0.01N $HClO_4$ containing 10 per cent. YEPW, and (2) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode.

D. Current-voltage curves for deoxygenated (1) 0.001N HCl containing 10 per cent. YEPW, and (2) 6.4×10^{-4}M cysteine HCl in 0.001N HCl containing 10 per cent. YEPW, using a saturated calomel reference electrode.

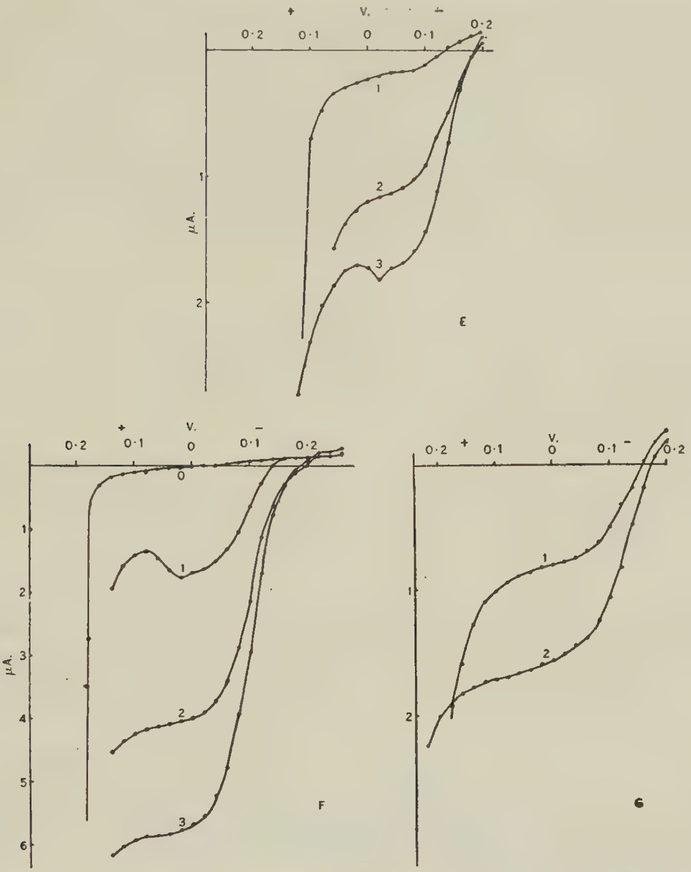

Fig. 3.—*E.* Current-voltage curves for deoxygenated (1) YEPW at pH 3, (2) (3) after autoclaving, and (3) 6.4×10^{-4}M cysteine HCl in YEPW at pH 3, using a saturated calomel reference electrode.

F. Current-voltage curves for deoxygenated (0) 0.01N $HClO_4$ containing 10 per cent. YEPW, (1) 6.4×10^{-4}M cysteine HCl in 0.01N $HClO_4$ containing 10 per cent. YEPW, (2) 10.5×10^{-4}M thioglycollic acid in 0.01N $HClO_4$ containing 10 per cent. YEPW, and (3) 6.4×10^{-4}M cysteine HCl and 10.5×10^{-4}M thioglycollic acid in 0.01N $HClO_4$ containing 10 per cent. YEPW, using a saturated calomel reference electrode. *G.* Current-voltage curves for deoxygenated (1) 3.2×10^{-4}M glutathione in 0.001N HCl, and (2) 6.4×10^{-4}M glutathione in 0.001N HCl, using a saturated calomel reference electrode.

Plotting the rate of oxidation of thioglycollic acid in YEPW.—During the course of experiments reported in a later section of this paper the concentration of thioglycollic acid varied from 1000 to 0 p.p.m. During the initial stages measurements were made by diluting the medium 1/10 in 0.01N $HClO_4$, thereby reducing the pH to *c.* 3 and preventing further oxidation. The 10 ml. sample was deoxygenated with nitrogen and the polarogram plotted over the range + 0.1 to — 0.1 V. (*v.* S.C.E.). This precaution was always taken to ensure that the wave had not altered its position. The concentration of thioglycollic acid could then be determined from the reading at + 0.02 V. *v.* S.C.E.

When the reading for the 1/10 dilution was reduced to 2-5 mm. samples of the medium were acidified to pH 3 with 1N HCl, deoxygenated, and polarograms plotted in the same manner, final readings being taken at — 0.02 V. *v.* S.C.E.

(*b*) *Cysteine*

Following upon observations made on thioglycollic acid the cysteine waves were examined in the following supporting electrolytes:

(i) 0.01N $HClO_4$ (Fig. 3*A*),
(ii) 0.001N HCl (Fig. 3*B*),
(iii) 0.01N $HClO_4$ containing 10 per cent. YEPW (Fig. 3*C*),
(iv) 0.001N HCl containing 10 per cent. YEPW (Fig. 3*D*),
(v) YEPW acidified to pH 3 with HCl (Fig. 3*E*).

Although a proportional increase in the anodic wave at 0.02 V. *v.* S.C.E. was obtained in 0.001N HCl (Fig. 3*B*) the waves were most unsatisfactory. Waves illustrated in Figures 3*A*, 3*C*, and 3*D* are similar to those reported by Kolthoff and Barnum (1940) who have discussed the significance of the 'maximum' produced.

The waves illustrated in Figure 3*E* (3) and (2), were obtained before and after heating the cysteine in YEPW respectively. The heating caused some unidentified change which prevented the maximum formation. Dilutions of the heated medium in 0.01N $HClO_4$ resulted in smooth, sigmoid waves without the maximum shown in Figure 3*C*.

The maximum was also suppressed by mixing thioglycollic acid with cysteine in the unheated medium (Fig. 3*F* (3)).

Relationship between cysteine concentration and diffusion current.—The ratio of diffusion current to cysteine concentration was found to be constant over the range 0-200 p.p.m. (0.0013M) of cysteine HCl (Fig. 4). This ratio was the same in all electrolytes tested and was not affected by the presence of agar.

In studying the oxidation of cysteine in YEPW initial determinations were made on 1/10 dilutions in 0.01N $HClO_4$ which were deoxygenated and the current measured at 0.06 V. *v.* S.C.E. (Fig. 3*C*) after plotting the polarogram between 0.1 and — 0.1 V. *v.* S.C.E. When readings were reduced to 2-5 mm. further determinations were made directly on YEPW acidified to pH 3 and deoxygenated. Polarograms were plotted over the same range and readings taken at — 0.02 V. *v.* S.C.E. (Fig. 3*E*).

It must be emphasized that the actual position of a wave depends largely on the composition of the medium. Slight changes have been the cause of a slight shift in the potential of the limiting current. Plotting of the wave ensured that measurements were always taken at the correct potential.

(*c*) *Combined Measurement of Thioglycollic Acid and Cysteine*

Figure 3*F* shows the waves obtained with cysteine (1) and thioglycollic acid (2) alone and in a mixture (3). The waves of these two substances coincide and are additive. In addition the maximum of the cysteine wave is not produced in the mixture.

The measurement of this single wave does not permit the expression of results in terms of cysteine and thioglycollic acid independently but does permit the rate of the oxidation of the combined mixture to be determined.

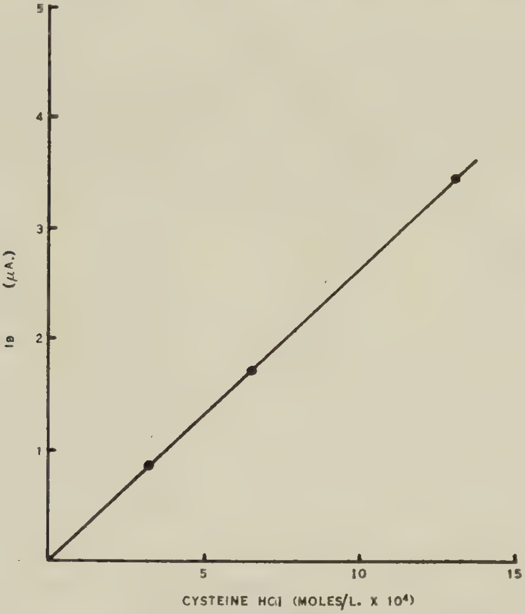

Fig. 4.—Diffusion current-cysteine HCl concentration calibration curve.

(*d*) *Glutathione*

The formation of an anodic depolarization wave by glutathione was examined in 0.001N HCl, in which it yields a clearly defined wave with the diffusion current proportional to the concentration over the range of 0-3 × 10^{-4}M. Two waves are shown in Figure 3*G*. Similar waves are obtained in YEPW with the diffusion current measurable at 0.0 or —0.02 V. *v.* S.C.E. (cf. cysteine).

(*e*) *Effect of By-products on the Calibration of the Polarograph for Measuring Cysteine and Thioglycollic Acid*

It seemed possible that the accumulation of cystine and dithioglycollic acid in the medium during the course of oxidation might affect the residual current measurements. Dithioglycollic acid was found to have no effect whatsoever. Cystine caused a change in portion of the residual current curve at potentials more negative than those employed in measuring cysteine but did not alter the residual current at the critical potentials.

(*f*) *Calibration of the Diffusion Currents in Terms of Sulphydryl Concentration*

It was intended that the nitroprusside test should be used in the titration of the sulphydryl with *p*-chlormercuribenzoate for the calibration of the polarograph. Parallel tests were conducted with the nitroprusside reagent against measurements made in the polarograph. Equal quantities of the various sulphydryl dilutions and 33 per cent. ammonia were mixed and 5 drops of a freshly prepared solution of sodium nitroprusside added. The colour change was observed up to 1 min. The results of these observations are tabulated in Table 1.

TABLE 1

COMPARISON OF NITROPRUSSIDE TESTS AND POLAROGRAPH MEASUREMENTS

—SH	Concentration (p.p.m.)	Galvanometer Deflection (mm.)	Initial Colour with Nitroprusside Reagent	Rate of Decolourization
Thioglycollic acid	1000 (0·012M)	1000	Purple	—
	100	100	Red	—
	50	50	Pink-red	—
	40	40	Pink	—
	20	20	Pink	+
	10	10	Pale pink	++
Cysteine HCl	400 (0·026M)	133	Pink	—
	200	67	Pink	+
	100	33	Pink	+++
	50	17	Yellow	

— = Not decolourized in 1 min.; + = decolourized in 1 min. or slightly less; ++ = decolourized in less than 30 sec.; +++ = transient colour only.

This table shows that the polarograph is capable of making quantitative estimations to a higher degree of sensitivity than that attainable with the nitroprusside test. With thioglycollic acid the sensitivity is only slightly greater but with cysteine hydrochloride a negative nitroprusside test is obtainable, particularly in YEPW, with concentrations up 150 p.p.m. For this reason an alternative method was sought for estimation of the sulphydryl by means of an amperometric titration using the polarograph itself. It was found that thioglycollic could be estimated by titration with mercuric chloride in 0.001N HCl provided the mercuric chloride was added to excess thioglycollic acid and not vice versa. Quantities of thioglycollic acid yielding diffusion currents of *c.* 3 μA. (66 mm. deflection on the galvanometer) were added to 0.001N HCl and the actual diffusion current measured at + 0.06 V. (see Fig. 1*C*). When quantities of 0.025 per cent. mercuric chloride were added to the thioglycollic acid the decrease in diffusion current was found to be strictly proportional to the amount of mercuric chloride added. Values given in Figure 2 were estimated in this way.

Reaction between the thioglycollic acid and mercuric chloride was instantaneous and there was no evidence of the formation of the highly insoluble mercurous salt.

When thioglycollic acid is added to excess mercuric chloride a white precipitate of mercurous salt is initially formed. It was not possible to use this method in the polarograph.

A similar attempt to estimate cysteine by the same procedure as that employed for thioglycollic acid was not successful. The titration could not be performed in HCl for reasons already indicated (see Fig. 3*B*). When an attempt was made to titrate in perchloric acid the changes in diffusion current with successive aliquots of mercuric chloride were not equal and a series of complicated anodic waves was encountered. The concentrations given for cysteine in Figure 4 were obtained gravimetrically. This should introduce very little error since the dry acid salt is quite stable. Fresh solutions in perchloric acid showed no evidence of the cathodic wave obtainable for cystine at more negative potentials.

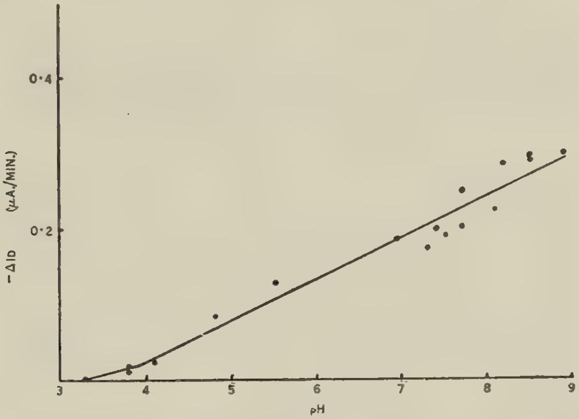

Fig. 5.—Effect of pH on the rate of reduction of oxygen concentration in YEPW by 0.1 per cent. thioglycollic acid expressed as rate of decrease in the oxygen diffusion current in μA. per min.

III. Observations on the Oxidation of Thioglycollic Acid and Cysteine in YEPW

(*a*) *Effect of pH on the Rate of Oxygen Reduction by* 0.1 *per cent. Thioglycollic Acid and* 0.1 *per cent. Cysteine HCl in YEPW*

In both cases 2 per cent. solutions of the reagents were prepared in glass-distilled water in sealed bottles. The quantity of 1N/NaOH required to neutralize 1 ml. of each was determined with a glass electrode and the concentration of the NaOH adjusted so that this amount was obtained in 1 ml.

The pH of the YEPW was adjusted to the desired value and 18 ml. placed in the cell, 1 ml. of the alkali added followed by 1 ml. of the reducing agent.

The rate of oxygen reduction (—△Id) was plotted at 1-min. intervals for 10 min. and then the final pH was determined. The rate was not constant but diminished with time. The curves obtained were therefore not linear.

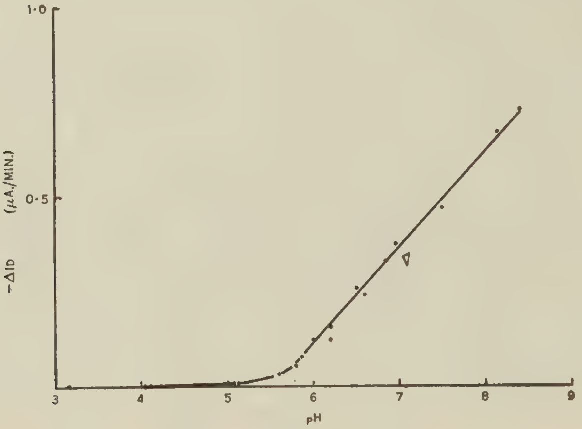

Fig. 6.—Effect of pH on the rate of reduction of oxygen concentration in YEPW by 0.1 per cent. cysteine HCl expressed as rate of decrease of the oxygen diffusion current in μA. per min.

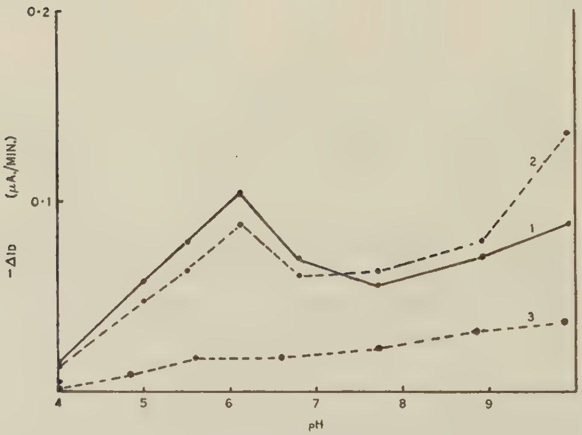

Fig. 7.—Curves (1) and (2) show the rate of reduction of oxygen (1) and sulphydryl (2) concentrations at varying pH values when 0.01 per cent. thioglycollic acid is added to YEPW saturated with oxygen at 37°C. Curve (3) shows the rate of reduction of sulphydryl concentration in the deoxygenated medium. All rates are expressed as rates of decrease in diffusion currents in μA. per min.

Figures 5 and 6, in which the tangents to the curves 2 min. after the addition of the reducing agent are plotted against pH, represent results obtained with thioglycollic acid and cysteine respectively. The 2-min. interval was chosen to allow turbulence caused by mixing of the reagents adequate time to subside.

No attempt was made to trace the rate of oxidation of the sulphydryls in these experiments. The complete removal of oxygen from the YEPW after the latter had been equilibrated with air at 30°C. caused too small a change in sulphydryl concentrations at the concentrations employed to permit any accurate analysis.

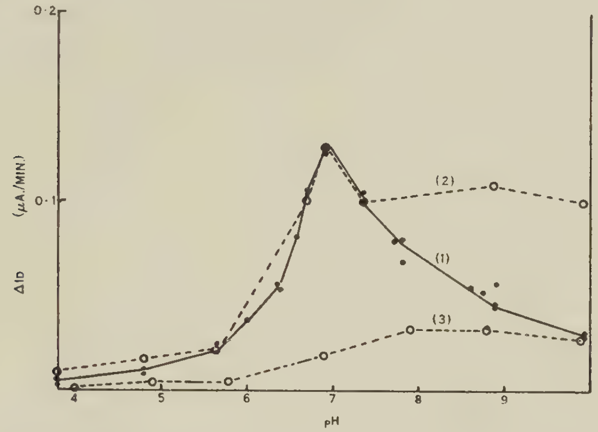

Fig. 8.—Curves (1) and (2) show the rate of reduction of oxygen (1) and sulphydryl (2) concentrations at varying pH values when 0.01 per cent. cysteine HCl is added to YEPW saturated with air at 37°C. Curve (3) shows the rate of reduction of sulphydryl concentration in the deoxygenated medium. All rates are expressed as rates of decrease in diffusion currents in μA. per min.

(*b*) *Effect of pH on the Rate of Reduction of Oxygen and Rate of Oxidation of* 0.01 *per cent. Cysteine and* 0.01 *per cent. Thioglycollic Acid in YEPW*

Previous unpublished experiments have shown that *Clostridium perfringens, Cl. tetani,* and *Cl. sporogenes* are all capable of growth in YEPW when inoculated after the concentration of reducing agents had fallen to 0.01 per cent. The behaviour of the reducing agent at these levels is therefore of considerable interest.

The small change in pH associated with the addition of such small quantities of acid obviated the necessity to take any special precaution to neutralize them on addition to the medium. Solutions (0.1 per cent.) were prepared in 0.001N HCl. Aliquots of 5 ml. of each were added to 45 ml. of YEPW and the rate of oxygen reduction (— △Id) plotted at 1-min. intervals for 4 min. At the 5-min. interval a 10-ml. sample was removed and the pH immediately adjusted to pH 3 by the addition of 0.25 ml. 1N HCl. The sample was deoxygenated and analysed for residual sulphydryl.

The rate of oxygen reduction was plotted at 1-min. intervals for a further 10 min., when a second sample was removed for residual sulphydryl estimation. The final pH was determined on the remaining broth.

In this manner the amount of sulphydryl oxidized to oxygen reduced could be estimated. The results are shown in Figures 7 and 8. In both figures the rate of reduction of oxygen (1) and oxidation of sulphydryl (2) are plotted as rate of change in their respective diffusion currents in μA. per min.

Provided the sulphydryl reduces only the oxygen in the medium or reduces oxygen and some other component at a uniform rate over the time interval employed the curves should be parallel. Any sharp increase in the rate of sulphydryl oxidation over oxygen reduction indicates a preferential reduction of some component other than oxygen in the medium.

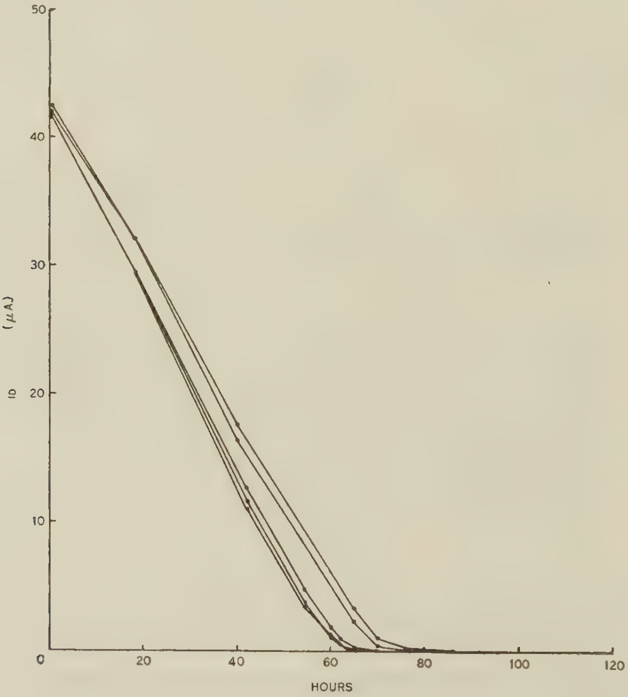

Fig. 9.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. $1\ \mu\text{A.} \equiv 2.6 \times 10^{-4}$ moles/l.

For cysteine (Fig. 8) the curves coincided over the range pH 5-7.3. The curves should have been parallel. Their coincidence is entirely fortuitous. Calculations show that the cysteine oxidized is entirely accounted for by the oxygen reduced. At higher pH values the cysteine appears to be preferentially oxidized by some other component of the medium. The rate of reaction with oxygen reached a maximum at the isoelectric point (pH 6.86).

Figure 8 (3) shows the rate of cysteine oxidation in the deoxygenated medium.

With thioglycollic acid the curves 1 and 2 remain parallel over the range pH 5-9. In the range pH 5-7.5 the amount of oxygen reduced exceeded the calculated by approximately 30 per cent. It approached the theoretical value between pH 7.5 and 9. Beyond this point the amount of sulphydryl oxidized greatly exceeded that calculated for the amount of oxygen reduced. No satisfactory explanation can be given for these findings. Similar results were obtained in nine replications of the experiment. It could not be explained by a change in the ratio of diffusion current to concentration of reactants at varying pH values.

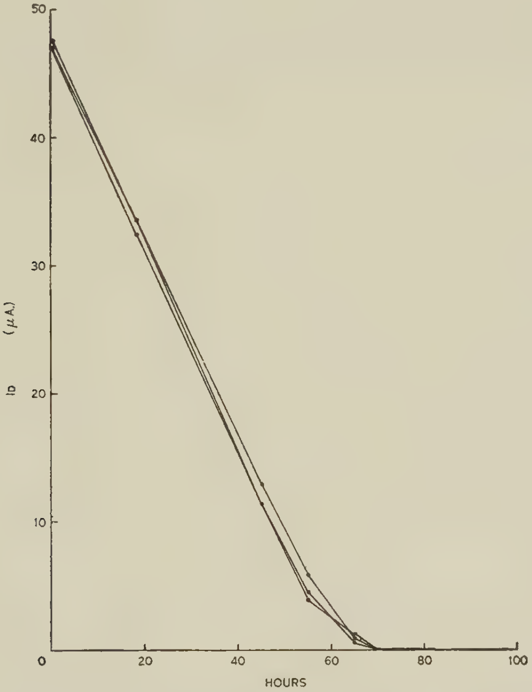

Fig. 10.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 2.6 × 10^{-4} moles/l.

(*c*) *Oxidation of Thioglycollic Acid and Cysteine in YEPW and other Media*

Brewer (1940) claimed that pork infusion containing 0.1 per cent. agar is a suitable medium for the cultivation of anaerobes. His final medium contained in addition thioglycollic acid and glucose although, from the tables supplied, the addition of these substances does not appear to have improved the medium. Since pork infusion *per se* did not indicate the presence of any specific reducing substance the initial step in the investigation was to study the oxidation of the thioglycollic acid in various media.

Thioglycollic acid (to 0.1 per cent.) was added to the medium and the pH adjusted to pH 7.0. The medium was dispensed in 330-350 ml. quantities in tall 800 ml. beakers to give a depth of 7 cm. The beakers were loosely plugged with cotton wool. A length of ½-in. glass tubing through which samples could be withdrawn was placed through the centre of the plug and covered with an aluminium cap. Care was taken in the preparation of the plugs to see that replicates were as uniform as possible since some earlier work has shown that the tightness of the plug was an important factor in the rate of oxidation through its ability to regulate the freedom of air movement. The flasks were autoclaved at 120°C. for 20 min., cooled, and placed in a 37°C. water bath with the water level ¼ in. above the level of the liquid in the beaker to minimize convectional movements. Analyses of the contents for residual — SH were conducted at intervals determined by the trend of the oxidation itself.

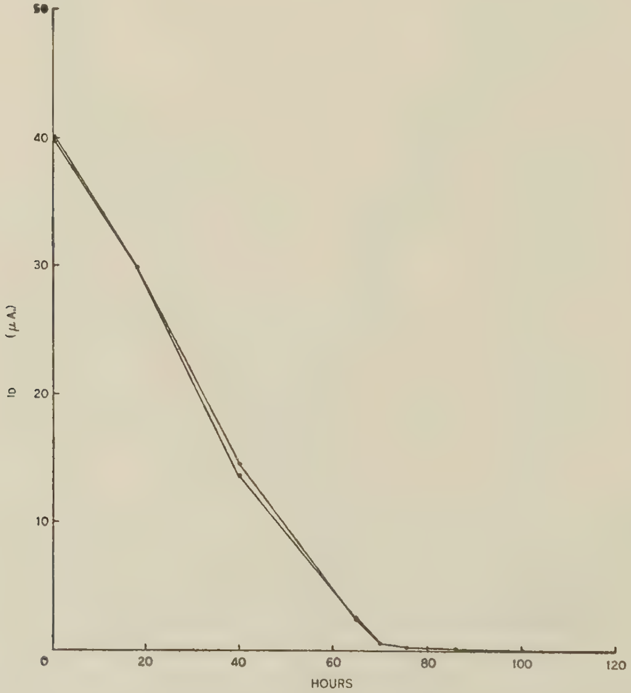

Fig. 11.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 1.0 per cent. glucose (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 2.6 × 10^{-4} moles/l.

The rate of oxidation of thioglycollic acid was studied in the following media:

(i) YEPW alone (Fig. 9),
(ii) YEPW + 0.1 per cent. agar (Fig. 10),
(iii) YEPW + 1.0 per cent. glucose (Fig. 11),
(iv) YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (Fig. 12).

Reference to these figures will show that 0.1 per cent. thioglycollic acid is completely oxidized in 70-80 hr. in YEPW and that the addition of agar and glucose had no detectable influence on this rate.

Comparative experiments in which *Clostridium perfringens, Cl. tetani,* and *Cl. sporogenes* were tested for their ability to grow in YEPW + 0.1 per cent. thioglycollic acid showed that growth of *Cl. perfringens* would occur in the medium up to and slightly beyond the point where approximately 0.005M thioglycollic acid (Id = 2 μA.) was left. *Cl. sporogenes* ceased growth approximately at this point and *Cl. tetani* slightly before it.

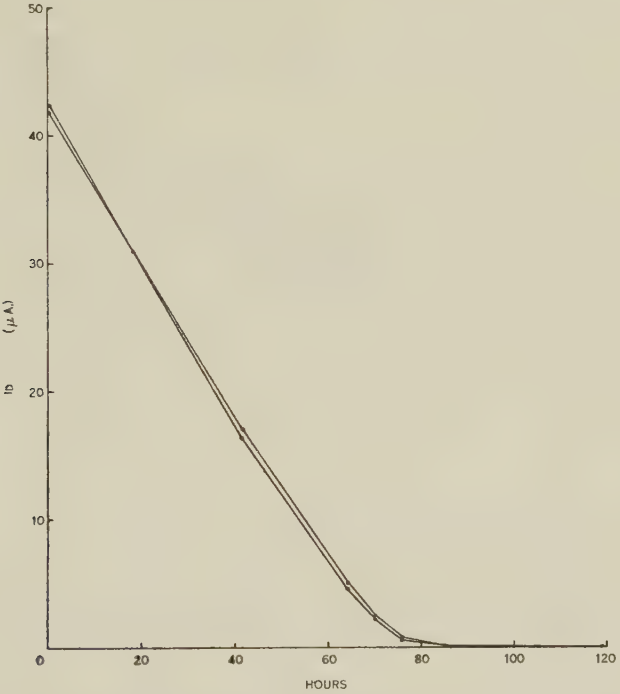

Fig. 12.—Rate of oxidation of 0.1 per cent. thioglycollic acid in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 2.6×10^{-4} moles/l.

Other experiments conducted with *Cl. perfringens* in YEPW, thioglycollic acid, and agar showed that agar prolonged the period of growth for 24-48 hr. beyond the complete oxidation of the thioglycollic acid.

None of these observations suggested why the Brewer medium should have remained effective for the period claimed. An examination was made therefore of pork infusion for the presence of an anodic wave suggestive of a sulphydryl compound. Minced pork in the proportion of 500 g./l. was steamed for 2 hr. in 0.5 per cent. NaCl. The hot infusion was filtered and a sample rapidly cooled, acidified to pH 3 with 1N HCl, deoxygenated with nitrogen, and a polarogram plotted. An anodic wave similar to that obtained with cysteine and thioglycollic acid in YEPW was obtained and the presence of a sulphydryl group was confirmed with the nitroprusside reagent.

Curves were plotted on similar extracts from ox liver, ox muscle, and veal. These curves are illustrated in Figure 13. It seems likely that the substance produced was cysteine, probably as glutathione (Hopkins 1929; Kendall, McKenzie, and Mason 1929). The possibility that this may have contributed to the prolonged effectiveness of Brewer's medium led to the investigation on the determination of cysteine and glutathione reported in an earlier section of this paper.

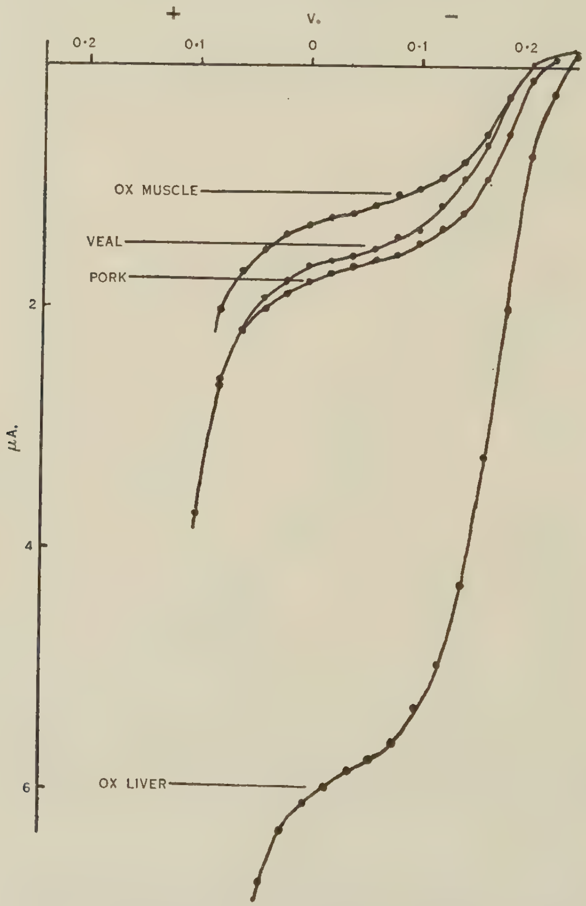

Fig. 13.—Sulphydryl waves obtained with tissue extracts prepared by steaming 50 per cent. w/v suspension for 2 hr., acidifying to pH 3 with HCl, and deoxygenating with nitrogen.

Owing to the high cost of glutathione only a single experiment was conducted with it. A 0.1 per cent. solution in YEPW at pH 7.0 equivalent in sulphydryl content to 0.05 per cent. cysteine HCl, was completely oxidized in 26 hr. at 37°C.

The oxidation of 0.1 per cent. cysteine HCl was studied in the following media: (i) YEPW alone (Fig. 14); (ii) YEPW + 0.1 per cent. agar (Fig. 15); (iii) YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (Fig. 16).

Cysteine is initially rapidly oxidized until approximately 60 p.p.m. are left. The remainder is very slowly oxidized. Growth of *Cl. perfringens* ceases at the point where rapid oxidation ceases (20 hr.) in the absence of agar and 24-48 hr. later in the presence of agar.

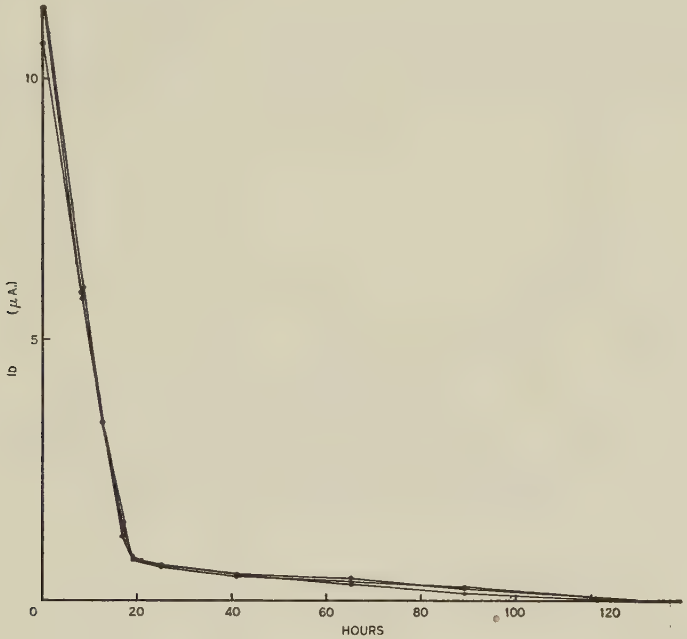

Fig. 14.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

The results of duplicate experiments in which 0.1 per cent. thioglycollic acid, 0.1 per cent. cysteine HCl, 1.0 per cent. glucose, and 0.1 per cent. agar were added to YEPW are shown in Figure 17. They are somewhat remarkable. It was expected that the time taken for oxidation of the combined sulphydryls would at least equal the sum of both. Instead the time barely exceeded that for thioglycollic acid alone and the slow process of oxidation of cysteine alone was completely absent.

Examination of pork infusion alone yielded results similar to those obtained with YEPW + 0.1 per cent. cysteine HCl.

(*d*) *Oxidation of Sulphydryl Groups in "Difco" Brewer's Medium and "Difco" Liquid Thioglycollate Medium*

In view of the results reported in the foregoing sections samples of the dehydrated "Difco" media used for sterility testing were obtained, prepared

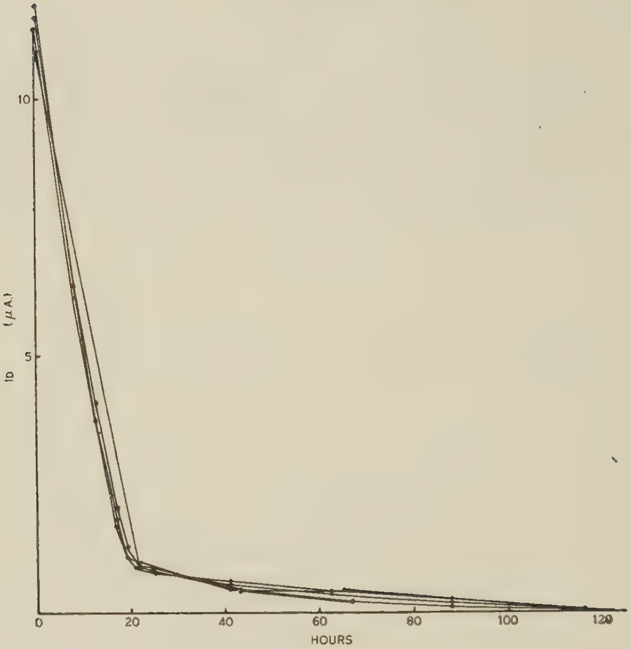

Fig. 15.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

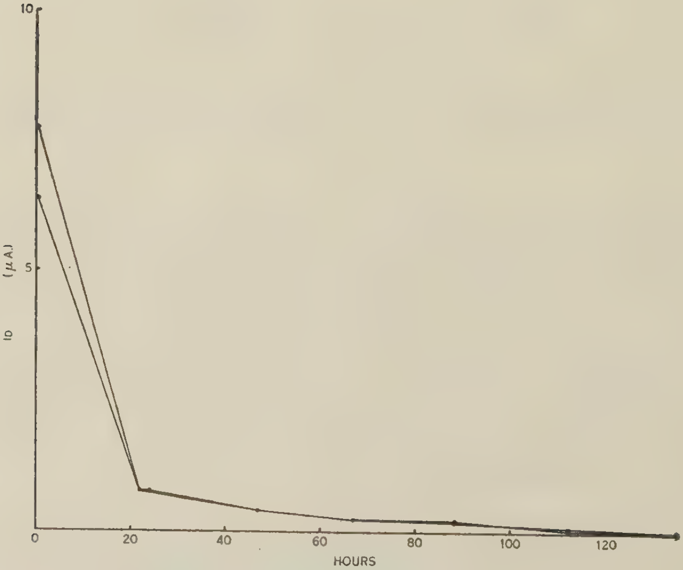

Fig. 16.—Rate of oxidation of 0.1 per cent. cysteine HCl in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm. 1 μA. ≡ 3.6×10^{-4} moles/l.

according to directions, and subjected to a similar course of study. The results are shown in Figures 18 and 19.

It was impossible with these media to determine the residual current for the — SH-free base medium. The anodic waves in the freshly autoclaved media occurred in the same position as that obtained for thioglycollic acid and cysteine in YEPW. The change in diffusion current was plotted to at least 100 hr. beyond the time when the media failed to support the growth of *Cl. perfringens.*

Neither of these media showed any better performance than those already reported. The idea that the prolonged action of these media was due to some mechanism that preserved a relatively high sulphydryl concentration had to be abandoned and the answer sought in some other direction. Two possibilities existed:

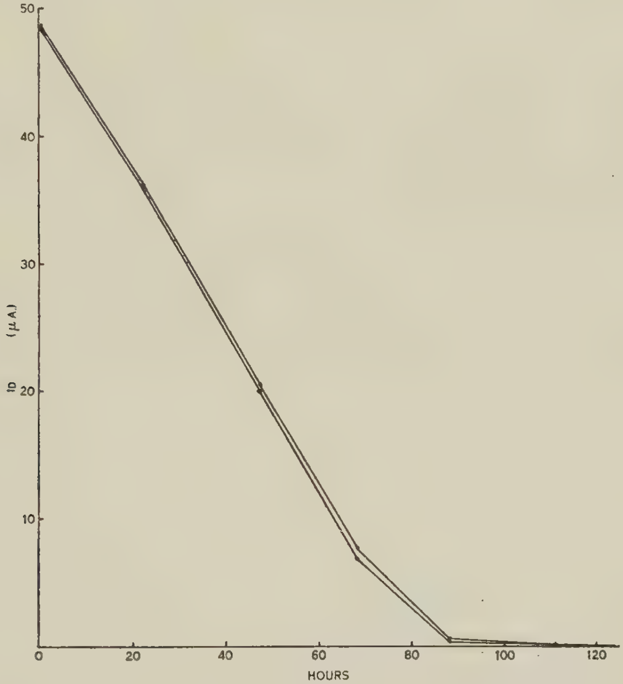

Fig. 17.—Rate of oxidation of a mixture of 0.1 per cent. thioglycollic acid and 0.1 per cent. cysteine HCl in YEPW + 1.0 per cent. glucose + 0.1 per cent. agar (pH 7.0) exposed to air at 37°C. in a depth of 7 cm.

(i) That the prolonged action was due to the method of dispensing, or

(ii) That the commonly advised procedure of boiling the medium prior to inoculation to "exclude dissolved oxygen" caused a regeneration of the sulphydryl.

That the method of dispensing is an important factor cannot be denied. "Difco" Liquid Thioglycollate medium dispensed in cotton-wool-plugged tubes in 7 cm. depth turns pink in less than 24 hr. and will support growth of *Cl. perfringens* for little more than 100 hr. if it is not reheated. Dispensed in screw-capped bottles it retains its yellow colour and effectiveness for months provided the seals are good.

Instructions are given to users of the medium to the effect that, once the medium has become oxidized to a point where it will no longer support anaerobic organisms, it can be reheated to remove dissolved oxygen and re-used.

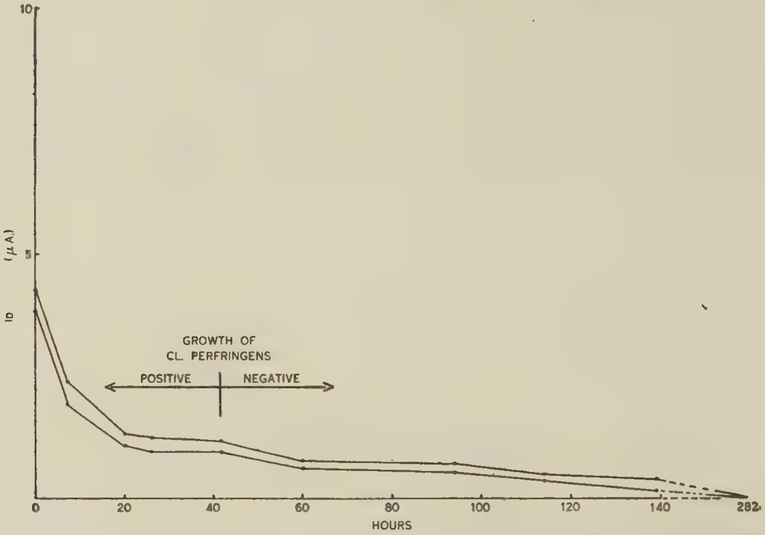

Fig. 18.—Rate of oxidation of sulphydryls in "Difco" Brewer's Medium.

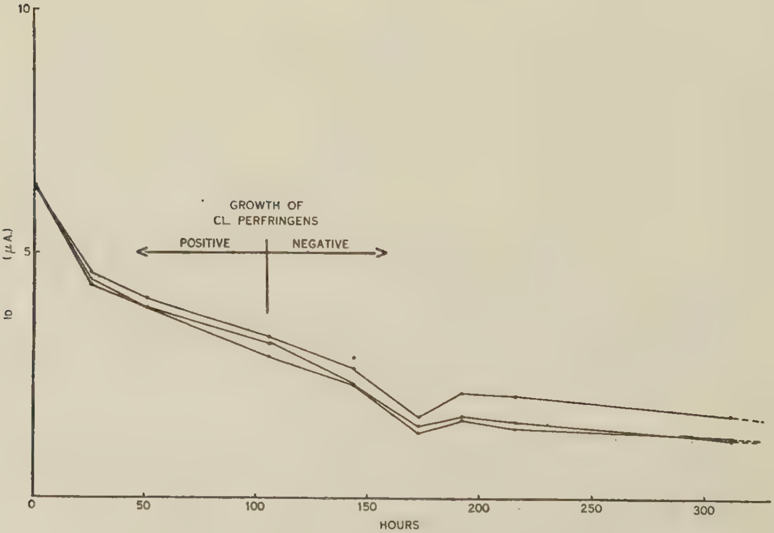

Fig. 19.—Rate of oxidation of sulphydryls in "Difco" Liquid Thioglycollate Medium.

It has been found that when YEPW is deoxygenated with nitrogen and exposed freely to air at a depth of 7 cm. at 37°C. without agitation, reoxygenation occurs to saturation level in a period of 8-10 hr. Media which have been reheated remain suitable for anaerobic culture for longer periods than this. It seemed logical to assume that heating regenerated the sulphydryl group. This supposition was put to test and found to be true.

Regeneration of — SH groups from disulphides in oxidized culture media.— Heating samples of YEPW containing the oxidation products of cysteine, thioglycollic acid, and glutathione at 121°C. for 20 min. at pH 7.0 resulted in regeneration of sulphydryl equivalent, on an average, to 20 per cent. of the initial sulphydryl content. According to Shinohara and Kilpatrick (1934) the process is one of hydrolysis. They demonstrated that the degree of hydrolysis increases with a rise in pH and is dependent on time. These observations have been substantiated by the author. The process is accelerated by constituents of the medium.

When cystine is autoclaved in the presence of free thioglycollic acid, as in "Difco" Liquid Thioglycollate Medium, the cathodic reduction wave (Kolthoff and Barnum 1941) for this substance disappears. It is only partially reduced in the absence of the sulphydryl compound under similar conditions. It seems possible therefore that the process may not be one of simple hydrolysis.

IV. Discussion

From the data presented it is abundantly clear that the suitability of Brewer's and similar media for the cultivation of anaerobic bacteria is dependent on the presence of free sulphydryl groups. Once these have been oxidized the medium will no longer support growth. Subsequently, heating of the medium will result in a regeneration of portion of the sulphydryl content as well as removal of the oxygen.

Glucose does not aid the establishment of anaerobic conditions. Its principal function is probably as a ready source of fermentable carbohydrate which would aid in the rapid establishment of a bacterial population.

Agar is claimed by Hitchens (1921) to support the growth of several types of organisms, both aerobic and anaerobic. In sulphydryl culture media it does not affect the rate of sulphydryl oxidation, and direct experiments (unpublished data) have shown that it has no effect on the rate of diffusion of oxygen in a concentration of 0.1 per cent. This supports the findings of Rahn and Richardson (1941). The action of agar is most noticeable during and after the final stage of oxidation of the sulphydryl. Where duplicate tubes have been inoculated and only one has shown growth it can be demonstrated, as pointed out by Hitchens, that growth occurs initially on small particles of separated agar. It has been the author's experience that such separation only occurs in tubes where growth occurs and would appear to be initiated by the cells themselves. Attempts to demonstrate that the separated agar had adsorbed and concentrated small quantities of sulphydryl have so far been abortive.

The author cannot support Brewer's claim that his medium is suitable for the support of anaerobes after exposure to air for one month. It seems likely that the medium has been reheated prior to inoculation.

In examining the large volumes of fluid for residual sulphydryl, samples were removed from the base of the flask as this was considered to be the region most significant in relation to growth of organisms in open tubes. This was based on the assumption that an oxygen gradient would exist from the surface to the base of the solution, the fluid at the base remaining deoxygenated for the longest period of time. Since the samples had to be acidified immediately on removal, it was not possible in these experiments to determine the oxygen concentration in the sample at the same time. When it was demonstrated that growth of selected anaerobes continued after the concentration of residual thioglycollic acid had reached 0.001M, a method was devised whereby it was possible to determine simultaneously both the concentration of sulphydryl and the oxygen in the medium and, at the same time, to determine the distribution of oxygen throughout the solution. The method and observations resulting from its application will be published in a subsequent paper. Pertinent to the present discussion are the findings that:

(i) The medium remains completely deoxygenated from a distance of 2 mm. and probably less from the surface from shortly after the addition of 0.01M thioglycollic acid until the concentration of the latter is reduced to approximately 0.00025M;

(ii) Resolution of the oxygen commences in this region and that its concentration rises uniformly throughout the solution until saturation is reached for the prevailing temperature. No concentration gradient was detectable in the solution either of oxygen or residual sulphydryl.

The studies were made on a 7 cm. depth of fluid held perfectly still in a temperature-controlled bath with the water-level of the bath above that of the containing vessel. Convectional movement of the solution, if any, was not detectable by the electrodes, which are particularly sensitive to such movements.

From these observations and those of the nitroprusside test (Table 1) it would appear that a medium yielding a pink to pale pink or yellow colour with the nitroprusside reagent may contain oxygen and requires reheating prior to inoculation. No purpose would be served in heating media yielding a deep pink to red colour.

The use of the dye resazurin is not recommended. Its oxidation to pink resorufin which occurs very quickly in open culture is not an index of complete sulphydryl oxidation or a return of oxygen to the medium and its presence complicates the application of the nitroprusside test.

V. ACKNOWLEDGMENTS

My thanks are due to Mr. Noel Henry of the Brisbane General Hospital for the supply of "Difco" Brewer's medium used in this investigation, and to Miss Shirley Hean for considerable technical assistance.

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INACTIVATION OF GONADOTROPHINS

IV. THE EFFECT OF PERIODATE IONS AND "BLOOD-GROUP ENZYME" ON THE BIOLOGICAL ACTIVITY OF CHORIONIC GONADOTROPHIN

By W. K. Whitten*

[*Manuscript received November* 3, 1952]

Summary

Treatment of chorionic gonadotrophin with periodate ions modified the hormone so that it was no longer inactivated by influenza virus. The dose response curve of the treated hormone was less steep than for untreated, which precluded any accurate comparison. However, at low doses no loss was detected.

Chorionic gonadotrophin was inactivated by a preparation of "blood-group enzyme."

I. Introduction

In a previous paper (Whitten 1950) it was shown that serum gonadotrophin was rapidly, but not completely, inactivated by dilute solutions of potassium periodate. The residual activity was distinct from that of untreated hormone, since it was no longer inactivated by influenza virus and produced a greater degree of luteinization than comparable doses of the untreated hormone as judged by ovarian weight increase. However, no departure from parallelism between the dose response curves of the untreated and treated hormone was observed, and subdivision of the dose did not increase the response. This was the first indication that the follicle-stimulating and the luteinizing effects of serum gonadotrophin could be dissociated.

The biological activity of serum gonadotrophin is one which produces follicle growth. However, there is some luteinizing activity and the total effect is one which at appropriate doses in hypophysectomized animals closely resembles the normal sequence of events of follicle growth, rupture, and formation of corpora lutea. Chorionic gonadotrophin, on the other hand, does not produce follicle growth in the absence of endogenous pituitary hormones, but results in luteinization of existing follicles and a stimulation of the interstitial cells.

Chemically, these hormones are strikingly similar, as is evidenced by their inactivation by proteolytic enzymes, taka-diastase, and saliva (Abramowitz and Hisaw 1939; Evans and Hauschildt 1942; Whitten 1947; Rimington and Rowlands 1950), by receptor-destroying enzyme and influenza virus (Whitten 1948*a*, 1948*b*), and, as will be shown later, by the "blood-group enzyme" of Morgan (Whitten 1949; Friedmann 1949). Nevertheless, differences have been detected (Li, Simpson, and Evans 1939; Bischoff 1942, 1946) which show that chorionic

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gonadotrophin is more resistant to nitrous acid, ketene, and to heat inactivation.

In view of the interesting results with periodic oxidation of serum gonadotrophin, and since chorionic gonadotrophin similarly contains a large carbohydrate moiety (Gurin, Bachman, and Wilson 1940), the effect of periodate on the latter was studied.

II. Methods and Material

Chorionic gonadotrophin.—This preparation was kindly supplied by Organon, England, and contained 585 I.U. per mg.

Influenza virus.—Allantoic fluid from chick embryos which had been infected with adapted LEE-B was kindly supplied by Dr. A. Isaacs of the World Influenza Centre.

"Blood-group enzyme."—This preparation was kindly supplied by Dr. W. T. J. Morgan of the Lister Institute. The preparation was dissolved in saline and heated at 56°C. for 1 hr.

Periodate solutions.—Potassium periodate of A.R. purity was prepared fresh each day, such that the solution was M/500 in M/15 phosphate buffer pH 6.0.

The remainder of the reagents used have been previously described in this series.

Animals and assays.—The technique of hormone assaying was similar to that described in the British Pharmacopœia (1948). The animals used were hooded rats from the Medical Research Council colony at Mill Hill, and were within the weight range of 40-50 g.

The dose response curve for this colony was established using a standard preparation and four groups of 10 animals at the following dose levels: 3, 9, 27, and 81 I.U. The dose response curve exhibited a linear regression of high significance ($P < 0.001$), without significant departure from linearity and producing mean ovarian weights from 20 to 76 mg. The ratio of the slope of this curve to its standard error was low, and therefore fiducial limits of error were calculated.

Experimental procedure.—Chorionic gonadotrophin was prepared at the beginning of each experiment in distilled water containing a few drops of chloroform, and the solution stored at 2°C. Each day equal volumes of the hormone and periodate solutions were mixed and were allowed to react for 30 min. at room temperature. After this period excess periodate was reduced by the addition of glucose. Control preparations were made by mixing hormone solution with periodate which had previously been reduced by glucose.

III. Observations

Preliminary observations indicated that reduced periodate had no appreciable effect on chorionic gonadotrophin. An assay conducted with untreated hormone against hormone treated with reduced periodate gave percentage recovery 94.07 with fiducial limits ($P = 0.05$) 60.38 and 150.71.

(*a*) *Effect of Periodate on Chorionic Gonadotrophin*

The effect on chorionic gonadotrophin was then studied in two experiments, the results of which are given in Table 1. In each case the dose response curves did not depart significantly from parallelism, and it will be seen that there is no appreciable loss of activity.

TABLE 1

PERCENTAGE OF ACTIVITY OF CHORIONIC GONADOTROPHIN RECOVERED AFTER TREATMENT WITH EXCESS PERIODATE IONS FOR 30 MIN.

Type of Assay	Percentage Recovery	Fiducial Limits of Error $P=0\cdot01$
Four-point . .	94·1	59·1-239·0
Six-point . .	86·4	73·3-139·7

Two attempts were then made to improve the accuracy of the assay by increasing the dose interval, but this proved unsuccessful since the dose response curves were no longer parallel ($P < 0.001$). In each case, as shown in Table 2, the lower dose groups were not significantly different, even though significant ovarian growth did occur, whereas the responses to the higher doses of the treated hormone were significantly lower than those of the untreated hormone ($P < 0.001$). In all cases comparable uterine weight increases were observed.

TABLE 2

MEAN OVARIAN WEIGHTS OBTAINED FOR TWO STANDARD FOUR-POINT ASSAYS OF CHORIONIC GONADOTROPHIN TREATED WITH PERIODATE IONS AGAINST UNTREATED HORMONE

Assay	Dose (mg.)	Treatment	Mean Ovarian Wt. (mg.±S.E.)
1	0·045	Treated	19·7±0·95
	0·045	Untreated	20·8±0·80
	0·135	Treated	32·3±1·84
	0·135	Untreated	48·9±2·21
2	0·045	Treated	21·7±0·89
	0·045	Untreated	23·0±1·34
	0·135	Treated	35·2±2·27
	0·135	Untreated	52·4±3·12

(*b*) *Effect of Influenza Virus on the Treated Hormone*

In this experiment comparison of ovarian responses at a single dose level was made between animals injected with oxidized hormone, and similar hormone incubated with influenza virus. The mean ovarian weights for these groups were respectively 33.3 ± 2.7 mg. and 25.8 ± 1.4 mg., which are not significantly different, whereas both were significantly different ($P < 0.001$) from the response obtained with an equivalent amount of unoxidized hormone incubated with virus (mean ovarian weight 15.7 ± 1.6 mg.). It is probable that some inactivation of endogenous gonadotrophin occurred when the active virus was injected into the test animals, which may account for the slight but not significant decrease of the response to oxidized hormone with virus.

(*c*) *Effect of "Blood-group Enzyme" on Chorionic Gonadotrophin*

Since it has been shown that serum gonadotrophin is rapidly inactivated by the enzyme preparation of Morgan (Whitten 1949; Friedmann 1949), it was decided to examine the effect of this on chorionic gonadotrophin. The enzyme was used as previously described, and again the comparison was made at one dose level between untreated hormone incubated with boiled enzyme and hormone incubated with the active enzyme preparation. The mean ovarian responses obtained were respectively 47.0 ± 5.4 mg. and 11.0 ± 0.5 mg. Analysis of the ovarian responses showed a significant difference ($P < 0.001$); in addition the latter mean ovarian weight was not different from that of untreated animals (11.5 ± 0.4 mg.), which indicates complete inactivation of the hormone.

IV. Discussion

Gaarenstroom and de Jongh (1946) report that it is generally agreed that only one active component is contained in chorionic gonadotrophin. This is supported by the work of Claesson *et al.* (1948), who have prepared electrophoretically homogeneous chorionic gonadotrophin in crystalline form. In view of these findings it is interesting that the biological activity can be modified so that the dose response curve is no longer parallel to that of untreated hormone, while the preparation retains its characteristic gonadotrophic action as shown by ovarian and uterine weight increases. The close agreement of the responses at low doses makes it unlikely that differences in absorption from the injection site, excretion, inactivation, or adsorption by the ovary from the blood could account for the decreased slope. This suggests that the hormone has two or more functions which vary in their susceptibility to periodate. That which is responsible for initial ovarian development is unaltered, while subsequent growth is retarded. However, it must be kept in mind that the response measured in these assays is the result of synergism between chorionic gonadotrophin and endogenous pituitary hormone.

In the many studies on the reactions of chorionic gonadotrophin, only Bischoff (1946) has made any attempt to compare the dose response curves before and after treatment. His observation that the products of heat denaturation were of reduced biological activity, but qualitatively and quantitatively identical with the original hormone, contrasts with the above findings. The detection of such a change in slope, while its significance is not understood, emphasizes the necessity for two or more point assays in these investigations.

In view of the previous findings with serum gonadotrophin in which it was shown that the luteinizing component was virtually unaffected with periodate, it is interesting that chorionic gonadotrophin, which is considered normally an interstitial cell-stimulating hormone, should not be altered so drastically, and supports the reports referred to earlier that this hormone is the more stable.

That periodic oxidation should again protect a gonadotrophin against destruction by influenza virus suggests that it may be possible to alter the hormone molecule in such a way that biological activity is apparently increased because it is no longer inactivated or excreted in the usual way. Perhaps this is the explanation of the progonadotrophic action of some immune sera.

The similarity between serum and chorionic gonadotrophins is given emphasis by their reactions with the "blood-group enzyme." The significance of this finding must await the characterization of the components of this enzyme preparation.

V. Acknowledgments

I am indebted to Dr. A. S. Parkes, F.R.S., of the National Institute for Medical Research, Mill Hill, in whose laboratory this work was carried out.

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THE INFLUENCE OF THYROID FUNCTION ON THE ALLEN-DOISY DOSE RESPONSE LINE OBTAINED BY THE ADMINISTRATION OF OESTRONE AND OESTRADIOL-3,17β IN MICE

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Summary

Hypothyroidism significantly increases the slope of the dose response line obtained by the subcutaneous and intravaginal administration of oestrone and concomitantly decreases the M.E.D. Hyperthyroidism has the reverse effect. Hypothyroidism, however, has no influence on the dose response line obtained by the intravaginal administration of oestradiol-3,17β.

The slope of the dose response line for oestradiol-3,17β is significantly lower than the slope for oestrone.

The significance of these results to an understanding of the mode of action of oestrogens is discussed.

I. Introduction

A considerable literature has now accumulated on the interaction between the hormones of the thyroid gland and the ovary (cf. reviews of Salter (1940, 1950)). Recently Langham and Gustavson (1947) and Barker *et al.* (1950) studied the influence of thyroid function on the Allen-Doisy oestrogen test in rats. They claimed that the thyroid status of the animals influenced their sensitivity to oestrone. As they did not use the method of log dose-probit analysis (or its equivalents), their interpretation of the results is questionable. A superficial examination of the figures of Langham and Gustavson, however, revealed the possibility that the level of thyroid activity could influence the slope of the dose response line. It was decided to investigate the nature of the dose response line in hypo- and hyperthyroid mice, using both the subcutaneous and intravaginal methods of administration.

II. Oestrone

(*a*) *Materials and Methods*

(i) *Preparation of Oestrogen Solutions.*—Nut oil was used as the vehicle for subcutaneous administration and distilled water was used as the vehicle for intravaginal administration. The oily solutions were prepared by the method described by Emmens (1950*b*) and the aqueous solutions by the method described by Biggers (1951*a*). The oestrone employed was a product of Organon Laboratories.

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(ii) *Biological Assay.*—Two colonies (*T*1 and *T*2) of female albino mice bred in the Department were used in this part of the investigation. Colony *T*1 consisted of 240 animals and colony *T*2 of 300 animals. Fourteen days after ovariectomy they were given subcutaneously a priming dose of 1 μg. oestrone in nut oil. Seven days later each colony was randomly divided into two groups; one group was used to determine the dose response line resulting from subcutaneous administration, while the other group was used to determine the dose response line resulting from intravaginal administration. The results are shown in Table 1. The colonies were accepted as satisfactory for use in Allen-Doisy assays.

TABLE 1

TEST SLOPES OBTAINED FOLLOWING THE SUBCUTANEOUS AND INTRAVAGINAL ADMINISTRATION OF OESTRONE TO COLONIES *T*1 AND *T*2

Colony	Route	Slope	Standard Error of Slope
*T*1	Subcutaneous	5·93	0·68
	Intravaginal	1·50	0·25
*T*2	Subcutaneous	5·85	0·97
	Intravaginal	1·79	0·46

Colony *T*1 was used to study the effect of thyroxine and methyl-thiouracil on the oestrone dose response line, while colony *T*2 was used to study the effect of ^{131}I. The plan of each experiment will be described as the results are presented. A standard 2-injection technique was employed for both the subcutaneous and intravaginal tests and the general procedures of randomization and animal management were as described by Biggers (1951*a*).

(*b*) *The Modification of Thyroid Activity*

Three methods have been used to alter the thyroid activity:

(i) The injection of thyroxine;

(ii) The administration of a goitrogen, methyl-thiouracil; and

(iii) The injection of thyroid-lethal doses of radioactive iodine, ^{131}I.

(i) *Thyroxine Administration.*—The normal average thyroid secretion rate in mature female mice has been estimated at 0.40-1.10 μg. DL-thyroxine per day, depending on the strain used (Hurst and Turner 1948). This was estimated from the amount of thyroxine required to maintain normal thyroid weight in animals fed thiouracil. In general it is believed that ovariectomy leads to decreased thyroxine production (see Hurst and Turner 1948).

The following procedure was adopted. Before each test, the colony was randomly divided into two groups, one group being used as a control and the other rendered hyperthyroid. Hyperthyroidism was induced by the subcutaneous injection of 4 μg. DL-thyroxine (B.D.H.) in 0.05 ml. distilled water for four consecutive days. The adequacy of the dose was confirmed by the anoxia resistance test of Smith, Emmens, and Parkes (1947). The oestrogen test was commenced on the seventh day, and after the conclusion of a test the colony was rested for one week before the next series of thyroxine injections.

(ii) *Methyl-thiouracil Administration.*—Several studies have appeared on the action of goitrogens in mice. Thiouracil has been the most commonly used substance (Dalton, Morris, and Dubnik 1946; Hurst and Turner 1947, 1948; Waldo, personal communication to Hurst and Turner 1948; Gorbman 1947*a*; Dalton *et al.* 1950). Several strains of mice have been studied by these workers, namely Schwing, Rockland, C_3H, yellow A^y Rockland hybrids, A, C57, I, and dba. Thyroid enlargement was noted by all the above workers except Waldo who, studying C57 and dba strains, found little change. Freiesleben, Kjerulf-Jensen, and Schmith (1945) have used methyl-thiouracil and observed only moderate hyperplasia.

4-Methyl-2-thiouracil has been employed in this work; 0.1 per cent. was incorporated in the drinking water and administration of the substance was maintained continually throughout the experiment. Over a period of 6-8 months no deleterious effects have been noted in the colonies, in agreement with the observations of Gorbman (1947*a*). The mice were given methyl-thiouracil for 10-14 days before an assay was commenced. Gorbman (1947*a*) described histological changes in the thyroid indicative of thyroxine deficiency within 7 days from the start of treatment. Also it has been found that methyl-thiouracil effectively increases the thyroid weight in the strain of mice used in these experiments (see Table 2).

TABLE 2

THYROID WEIGHT OF UNTREATED MICE AND MICE HAVING RECEIVED 0.1 PER CENT. METHYL-THIOURACIL IN THE DRINKING WATER OVER A PERIOD OF 3 WEEKS

	Mean Thyroid Weight (mg.)	Standard Error of Mean
Untreated	5·60	0·30
Treated	16·38	1·47

(iii) *^{131}I Administration.*—The first report of the thyroid-lethal properties of ^{131}I was by Hamilton (1942) who showed the effect of 300 μc. ^{131}I per kg. body weight in a dog. The effect of massive doses of ^{131}I in mice has been extensively studied by Gorbman (1947*b*, 1949*a*, 1949*b*, 1950), who has shown that the dose of ^{131}I required to destroy the thyroid depends greatly on the iodine content of the diet. It appears that provided that 4.1 μc. ^{131}I per mg. of thyroid enters the gland the dose will be effective.

Colony *T*2 was randomized into two groups, one of 200 and the other of 100 mice. Each animal of the larger group received a single subcutaneous injection of 250 μc. ^{131}I while the animals of the smaller group were kept as controls. The ^{131}I was obtained from the Atomic Research Establishment, Harwell, England. It was received ready for injection, the carrier solution being sodium bisulphite, pH 9.1, which had no obvious irritant action.

(*c*) *Results*

(i) *Subcutaneous Administration.*—The results obtained in mice with thyroxine and methyl-thiouracil are shown in Table 3. The estimates of slope

Table 3

DOSE RESPONSE DATA FOR SUCCESSIVE TESTS OF OESTRONE IN OIL ADMINISTERED SUBCUTANEOUSLY TO UNTREATED MICE, AND MICE RECEIVING THYROXINE OR METHYL-THIOURACIL

Untreated					Treated			
Test No.	Dose (μg.)	No. of Animals per Group	Animals Positive (%)	Mean Slope	Dose (μg.)	No. of Animals per Group	Animals Positive (%)	Mean Slope
						Thyroxine		
1	0·037	19	10·5	5·33 ±0·40	0·037	19	15·8	3·34 ±0·76
	0·05	20	35		0·05	20	30	
	0·069	20	65		0·069	20	40	
	0·091	20	75		0·091	20	60	
	0·123	18	83·3		0·123	20	70	
2	0·037	25	0		0·037	25	8	
	0·05	25	32		0·05	25	24	
	0·069	25	40		0·069	25	20	
	0·091	25	64		0·091	24	50	
3	0·037	19	0		0·037	19	5·3	
	0·05	20	20		0·05	19	15·8	
	0·069	20	5		0·069	20	20	
	0·091	20	50		0·091	20	55	
	0·123	20	95		0·123	20	65	
						Methyl-thiouracil		
4	0·037	19	5·3		0·037	18	0	7·34 ±0·85
	0·05	19	10·5		0·046	19	10·5	
	0·069	19	36·8		0·058	19	5·3	
	0·091	19	63·2		0·073	19	57·9	
	0·123	19	89·5		0·091	18	83·3	
5	0·037	19	10·5		0·037	16	0	
	0·05	20	30		0·046	19	21·1	
	0·069	18	66·7		0·058	19	31·6	
	0·091	17	52·9		0·073	18	66·7	
	0·123	17	100		0·091	19	73·7	
6	0·037	18	5·6		0·037	18	0	
	0·05	18	16·7		0·046	18	5·6	
	0·069	18	33·3		0·058	18	27·8	
	0·091	18	66·7		0·073	18	55·6	
	0·123	18	88·9		0·091	18	72·2	

of the individual dose response lines are variable. Table 4 shows that while the *within mean slope* variations can be considered as due to random sampling, there are highly significant differences between the mean slopes obtained with the untreated, thyroxine-treated, and methyl-thiouracil-treated groups respectively. The results are illustrated in Figure 1. A comparison of the mean slope from the methyl-thiouracil group and that of the untreated group shows them to be significantly different ($\chi^2_{(1)} = 5.00$, $P < 0.05$). Similarly a comparison between the mean slopes obtained with the untreated and thyroxine-treated groups respectively shows a significant difference ($\chi^2_{(1)} = 9.57$, $P < 0.01$).

TABLE 4

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 1

Source of Variation	D.F.	χ^2	P
Parallelism:			
1. Between mean slopes ..	2	18·74	<0·001
2. Within mean slopes ..	9	8·38	0·7-0·5
Heterogeneity	28	40·58	0·1-0·05

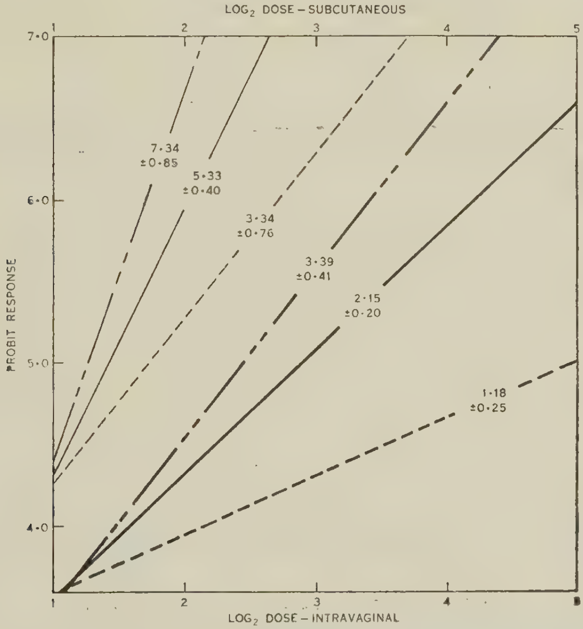

Fig. 1.—Dose response lines calculated from the data of Tables 3 and 9. Thin lines (upper), subcutaneous data. Thick lines (lower), intravaginal data; ——- - -—— hypothyroidism; ———— normal; – – – – – – hyperthyroidism.

An analysis of the M.E.D.'s is given in Table 5. The effect of thyroxine is to increase significantly the M.E.D. The effect of methyl-thiouracil appears to be in the reverse direction, i.e. it tends to decrease the M.E.D., but the result just fails to reach conventional significance.

TABLE 5

RATIO OF THE M.E.D.'S AND FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 2*

Test No.	Treatment	Log$_2$ M.E.D.	S.E. of Log$_2$ M.E.D.	Ratio M.E.D. of Untreated to M.E.D. of Treated	Fiducial Limits of Error ($P=0·05$) of Ratio
1	None	1·262	0·103	0·83	0·65-1·07
	Thyroxine	1·527	0·156		
2	None	1·436	0·106	0·75	0·51-1·11
	Thyroxine	1·857	0·266		
3	None	1·503	0·074	0·82	0·66-1·01
	Thyroxine	1·790	0·136		

Combined data for thyroxine: weighted mean ratio, 0.81; fiducial limits of error ($P = 0.05$), 0.70-0.95.

4	None	1·502	0·090	1·15	0·99-1·33
	Methyl-thiouracil	1·307	0·064		
5	None	1·244	0·123	1·05	0·87-1·25
	Methyl-thiouracil	1·181	0·083		
6	None	1·362	0·088	1·06	0·90-1·25
	Methyl-thiouracil	1·282	0·075		

Combined data for methyl-thiouracil: weighted mean ratio, 1.09, fiducial limits of error ($P = 0.05$), 0.99-1.19.

* In all figures and tables used in assessing results with the subcutaneous method logarithmic transformates, p, of the doses given, X_p, are used, in accordance with the formula $X_p = 2.7 \times 10^{-2} \times 1.35^p$.

The results obtained with Colony *T2* are shown in Table 6. Table 7 shows that while *within mean slope* variations can be considered as due to random sampling, there is a significant difference between the mean slope for the untreated group and the mean slope of the ^{131}I-treated group.

An analysis of the M.E.D.'s is given in Table 8. The effect of the ^{131}I is to decrease significantly the M.E.D., an effect which was on the borderline of significance with methyl-thiouracil (Table 5).

TABLE 6

DOSE RESPONSE DATA FOR SUCCESSIVE TESTS OF OESTRONE IN OIL ADMINISTERED SUBCUTANEOUSLY TO UNTREATED MICE AND MICE TREATED PREVIOUSLY WITH A THYROID-LETHAL DOSE OF ^{131}I

	Untreated				^{131}I			
Test No.	Dose (μg.)	No. of Animals per Group	Animals Positive (%)	Mean Slope	Dose (μg.)	No. of Animals per Group	Animals Positive (%)	Mean Slope
1	0·037	20	5	5·44 ±0·68	0·037	34	5·9	7·90 ±0·84
	0·050	20	10		0·046	34	8·8	
	0·069	28	25		0·058	40	22·5	
	0·091	20	50		0·073	41	65·9	
	0·123	20	85		0·091	34	82·4	
2	0·037	20	5		0·037	18	0	
	0·050	20	15		0·046	20	10	
	0·069	27	33·3		0·058	26	23·1	
	0·091	20	60		0·073	20	65	
	0·123	20	90		0·091	20	85	

TABLE 7

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 6

Source of Variation	D.F.	χ^2	P
Parallelism:			
1. Between mean slopes . .	1	5·19	0·05-0·02
2. Within mean slopes . .	2	0·60	0·8-0·7
Heterogeneity	11	6·86	0·9-0·8

TABLE 8

RATIO OF THE M.E.D.'S AND FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 6

Test No.	Treatment	Log_2 M.E.D.	S.E. of Log_2 M.E.D.	Ratio M.E.D. of Untreated to M.E.D. of Treated	Fiducial Limits of Error (P=0·05) of Ratio
1	None	1·655	0·091	1·38	1·19-1·61
	^{131}I	1·188	0·059		
2	None	1·517	0·088	1·25	1·18-1·44
	^{131}I	1·196	0·059		

Combined data: weighted mean ratio 1.31; fiducial limits of error ($P = 0.05$), 1.18-1.45.

(ii) *Intravaginal Administration.*—The results obtained in mice treated with thyroxine and methyl-thiouracil are shown in Table 9. Table 10 shows that while the *within mean slope* variations can be considered as random fluctuations, there are highly significant differences between the mean slopes obtained with the untreated, thyroxine-treated, and methyl-thiouracil-treated mice respectively.

TABLE 9

DOSE RESPONSE DATA FOR SUCCESSIVE TESTS OF OESTRONE IN DISTILLED WATER ADMINISTERED INTRAVAGINALLY* TO UNTREATED MICE, AND MICE RECEIVING THYROXINE OR METHYL-THIOURACIL

Untreated					Treated			
Test No.	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope
						Thyroxine		
1	0·1	20	5	2·15 ±0·20	0·1	20	5	1·18 ±0·25
	0·2	16	12·5		0·2	19	15·8	
	0·4	19	36·8		0·4	20	25	
	0·8	20	65		0·8	18	27·8	
	1·6	20	85		1·6	20	45	
2	0·1	18	11·1		0·1	20	5	
	0·2	20	20		0·2	19	15·8	
	0·4	18	50		0·4	20	30	
	0·8	19	68·4		0·8	20	35	
	1·6	18	88·9		1·6	19	52·6	
						Methyl-thiouracil		
3	0·1	18	11·1		0·1	19	10·5	3·40 ±0·41
	0·2	18	27·8		0·18	18	27·8	
	0·4	17	41·2		0·32	17	52·9	
	0·8	18	66·7		0·57	19	89·5	
	1·6	16	87·5		1·00	18	94·4	
4	0·1	18	11·1		0·1	16	0	
	0·2	15	26·7		0·18	19	15·8	
	0·4	18	55·6		0·32	19	52·6	
	0·8	15	80		0·57	19	89·5	
	1·6	19	94·7		1·00	18	94·4	

* In all figures and tables used in assessing results with the intravaginal method logarithmic transformates, p, of the doses given, X_p, are used, in accordance with the formula $X_p = 5.0 \times 10^{-5} \times 2^p$.

The results are illustrated graphically in Figure 2. A comparison of the mean slope from the methyl-thiouracil group and that of the untreated group shows them to be significantly different ($\chi^2_{(1)} = 7.29$, $P < 0.01$). Similarly a comparison between the mean slopes obtained with the untreated and the thyroxine-treated groups respectively also shows a significant difference ($\chi^2_{(1)} = 9.10$, $P < 0.01$). An analysis of the M.E.D.'s is given in Table 11. The effect of thyroxine is to increase significantly the M.E.D. while the effect of methyl-thiouracil is to decrease it significantly.

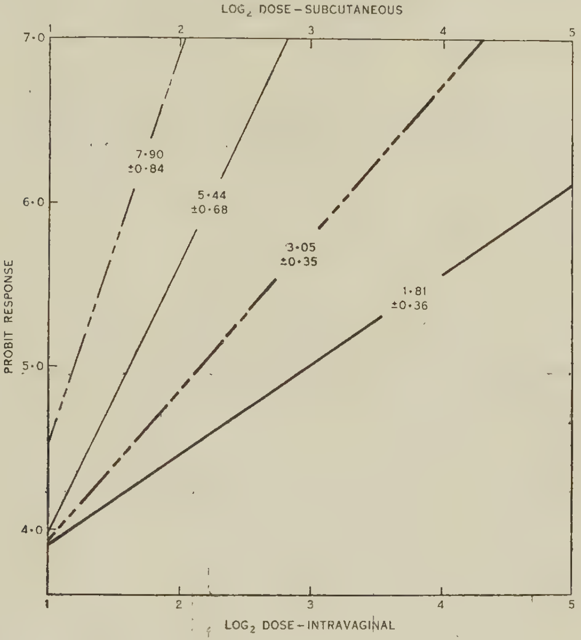

Fig. 2.—Dose response lines calculated from the data of Tables 6 and 12. Key as for Figure 1.

The results obtained in the ^{131}I mice are given in Table 12. They are analysed in Tables 13 and 14 and fully confirm the results obtained with methyl-thiouracil.

TABLE 10

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 9

Source of Variation	D.F.	χ^2	P
Parallelism:			
1. Between mean slopes ..	2	22·66	<0·001
2. Within mean slopes ..	5	1·76	0·9-0·8
Heterogeneity	23	4·68	>0·99

All slopes obtained in hypothyroid animals with oestrone over the experimental period have been combined and compared with the weighted mean slope for oestrone in normal animals (Biggers 1953). The results are shown in Table

15. Ignoring the slight heterogeneity in the distilled water group, a highly significant difference is seen between the two groups ($t = 6.16$, D.F. 50, $P < 0.001$). Thus the higher slope in hypothyroid animals with oestrone is confirmed.

Table 11

RATIO OF THE M.E.D.'S AND FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 9

Test No.	Treatment	Log$_2$ M.E.D.	S.E. of Log$_2$ M.E.D.	Ratio M.E.D. of Untreated to M.E.D. of Treated	Fiducial Limits of Error (P=0·05) of Ratio
1	None	3·925	0·219	0·36	0·11-1·11
	Thyroxine	5·417	0·812		
2	None	3·134	0·237	0·32	0·14-0·72
	Thyroxine	4·782	0·552		

Combined data for thyroxine: weighted mean ratio, 0.33; fiducial limits of error ($P = 0.05$), 0.17-0.64.

3	None	3·178	0·269	1·82	1·18-2·82
	Methyl-thiouracil	2·310	0·175		
4	None	2·785	0·225	1·19	0·83-1·71
	Methyl-thiouracil	2·538	0·144		

Combined data for methyl-thiouracil: weighted mean ratio, 1.41; fiducial limits of error ($P = 0.05$), 1.07-1.87.

III. OESTRADIOL-3,17β

As a result of the findings with oestrone it was decided to determine the relative potencies of other oestrogens in hypothyroid mice. Oestradiol-3,17β was selected for the first examination.

(*a*) *Materials and Methods*

Colony *T*3, consisting of 260 ovariectomized albino mice, was prepared for this work. Hypothyroidism was induced by the administration of methylthiouracil in the drinking water as before and all other methods were identical with those described for the oestrone experiments. The oestradiol-3,17β was a product of Organon Laboratories.

(*b*) *Results*

The design of the assays and the results obtained are shown in Table 16. Table 17 shows that very little difference exists *within mean slopes* while a highly significant difference exists *between mean slopes*. Thus no estimation of the relative potency can be made. However, Table 18 shows that the M.E.D. for oestradiol-3,17β is significantly lower than the M.E.D. for oestrone (see Fig. 3).

TABLE 12

DOSE RESPONSE DATA FOR SUCCESSIVE TESTS OF OESTRONE IN DISTILLED WATER ADMINISTERED INTRAVAGINALLY TO UNTREATED MICE AND MICE TREATED PREVIOUSLY WITH A THYROID-LETHAL DOSE OF ^{131}I

	Untreated				^{131}I			
Test No.	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope
1	0·1	21	9·5	1·81 ±0·36	0·10	30	10·0	3·05 ±0·35
	0·2	20	25		0·16	33	36·4	
	0·4	26	46·2		0·26	40	55·0	
	0·8	21	66·7		0·41	39	74·4	
	1·6	19	94·7		0·66	32	90·6	
2	0·1	19	21·1		0·10	18	16·7	
	0·2	21	38·1		0·16	19	42·1	
	0·4	26	50		0·26	25	64·0	
	0·8	15	73·3		0·41	18	88·9	
	1·6	20	85·0		0·66	17	94·1	

TABLE 13

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 12

Source of Variation	D.F.	χ^2	*P*
Parallelism:			
1. Between mean slopes . .	1	7·92	0·01-0·001
2. Within mean slopes . .	2	1·88	0·5-0·3
Heterogeneity	12	2·39	>0·99

The results show that the mean slope for oestradiol-3,17β is significantly lower than that of oestrone. This result could mean that the thyroid status of the animal has no effect on the dose response line for oestradiol-3,17β; this possibility was examined in the ^{131}I-treated colony. Table 19 shows the design of the assay and the results obtained. Tables 20 and 21 show that hypothyroidism has no influence on the dose response line obtained with oestradiol-3,17β.

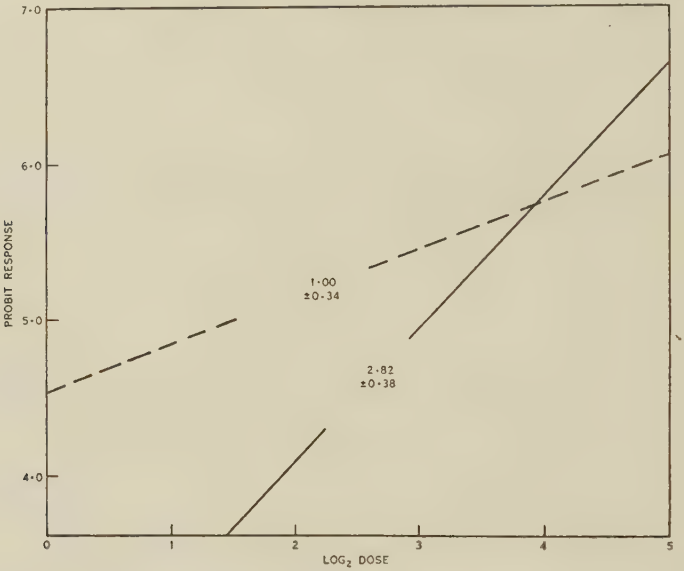

Fig. 3.—Dose response lines calculated from the data of Table 16. ------ Oestradiol-3,17β; ———— oestrone.

Table 14

RATIO OF THE M.E.D.'S AND FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 12

Test No.	Treatment	Log$_2$ M.E.D.	S.E. of Log$_2$ M.E.D.	Ratio M.E.D. of Untreated to M.E.D. of Treated	Fiducial Limits of Error (P=0·05) of Ratio
1	None	3·084	0·211	2·25	1·62-3·13
	^{131}I	1·910	0·120		
2	None	2·786	0·294	2·25	1·46-3·46
	^{131}I	1·618	0·156		

Combined data: weighted mean ratio, 2.25; fiducial limits of error ($P = 0.05$), 1.73-2.94.

IV. Discussion

The significant variations in the slope found between the oestrone dose response lines of normal, hypothyroid, and hyperthyroid mice are reflections of the significant differences in variability of threshold doses of oestrone required to produce cornification. Similarly differences in slope between the oestrone and oestradiol-3,17β dose response lines are reflections of the significant difference in variation of the threshold doses of the two steroids. The method of probit analysis is derived from considerations of the integrated normal distribution curve* (see Finney 1947, 1949). By changing the theoretical probit values

TABLE 15

A COMPARISON OF THE WEIGHTED MEAN OF THE SLOPES OBTAINED WITH NORMAL ANIMALS (50 PER CENT. AQUEOUS GLYCEROL AND DISTILLED WATER GROUPS) AND HYPOTHYROID ANIMALS

	Normal Group	Hypothyroid Group
Weighted mean slope	2·08	3·07
V_b (theoretical)	0·00543	0·0487
V_b (observed)	0·00913	0·0220
	$F=1{\cdot}68$, $n_1=44$	$F=2{\cdot}22$, $n_1=\infty$
	$n_2=\infty$	$n_2=6$
	$0{\cdot}01< P<0{\cdot}05$	$P>0{\cdot}05$

TABLE 16

DOSE RESPONSE DATA FOR SUCCESSIVE TESTS OF OESTRONE AND OESTRADIOL-3,17β IN DISTILLED WATER ADMINISTERED INTRAVAGINALLY TO MICE RECEIVING METHYL-THIOURACIL

	Oestrone				Oestradiol-3,17β			
Test No.	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope	Dose (μg. × 10^{-3})	No. of Animals per Group	Animals Positive (%)	Mean Slope
1	0·28	20	10	2·82 ±0·38	0·17	20	40	1·00 ±0·34
	0·5	20	50		0·3	19	57·9	
	0·9	20	65		0·54	20	55	
2	0·28	25	40		0·28	35	65·7	
	0·5	25	64		0·5	25	80	
	0·9	25	88		0·9	25	84	
3	0·1	25	4		0·1	24	58·3	
	0·2	25	24		0·2	25	68	
	0·4	25	52		0·4	25	88	
	0·8	25	80		0·8	24	83·3	

---

* There are no *a priori* reasons for the assumption of a normal distribution of threshold doses of oestrogen, e.g. Biggers (1951*b*) has shown that other transformations describe the data equally well.

from the linear regression lines back to percentages the theoretical integrated normal curves are obtained. Differentiation of these curves gives the theoretical normal distribution of threshold doses. Figure 4 shows the theoretical distributions calculated from the intravaginal data for the thiouracil and thyroxine experiments and Figure 5 for the oestrone and oestradiol-3,17β experiments.

TABLE 17

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 16

Source of Variation	D.F.	χ^2	P
Parallelism:			
1. Between mean slopes ..	1	12·90	<0·001
2. Within mean slopes ..	4	0·34	0·99-0·98
Heterogeneity	8	7·61	0·5-0·3

TABLE 18

RATIO OF THE M.E.D.'S AND FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 16

Test No.	Treatment	$\log_2$ M.E.D.	S.E. of $\log_2$ M.E.D.	Ratio of M.E.D. of Oestrone to M.E.D. of Oestradiol-3,17β	Fiducial Limits of Error (P=0·05) of Ratio
1	Oestrone	3·500	0·198	2·19	0·77-6·23
	Oestradiol-3,17β	2·365	0·742		
2	Oestrone	2·675	0·220	2·84	0·56-14·3
	Oestradiol-3,17β	1·145	1·170		
3	Oestrone	2·946	0·182	6·63	1·37-32·1
	Oestradiol-3,17β	0·217	1·008		

Combined data: weighted mean ratio, 3.02; fiducial limits of error ($P = 0.05$), 1.40-6.50.

The demonstration of a difference between the dose response lines of oestrone and oestradiol-3,17β in intravaginal tests indicates a fundamental distinction between the *local* actions of these steroids on the vagina. This seems to be the first demonstration of a qualitative difference in the action of two natural oestrogenic steroids and of considerable importance in the problem of molecular configuration and activity. The results obtained by the subcutaneous administration of oestrone merely confirm the action of thyroxine on the dose response line shown by the intravaginal technique.

It seems that oestrone is in some way modified in the vagina whereas oestradiol-3,17β is not, and that the modification of oestrone is under the control of thyroid activity. There are several reports in the literature on the increase in oestrogenic activity observed when oestrone is incubated with various tissues. Mamoli (1938) demonstrated the conversion of oestrone esters to oestradiol-3,17β by fermenting yeast. Heller (1940) showed that oestrone activity was greatly enhanced by incubation with minced uterus and to a lesser extent by heart, lung, spleen, and kidney; liver, however, inactivated the hormone unless cyanide was added, following which increased activity was also

TABLE 19

DOSE RESPONSE DATA FOR A TEST OF OESTRADIOL-3,17β IN DISTILLED WATER ADMINISTERED INTRAVAGINALLY TO UNTREATED MICE AND MICE TREATED PREVIOUSLY WITH A THYROID-LETHAL DOSE OF ^{131}I

	Untreated			I^{131}		
Dose (μg. $\times 10^{-3}$)	No. of Animals per Group	Animals Positive (%)	Mean Slope	No. of Animals per Group	Animals Positive (%)	Mean Slope
0·05	20	40	1·40 ±0·45	20	45	0·92 ±0·43
0·1	20	40		18	50	
0·2	20	75		20	50	
0·4	20	80		20	80	

TABLE 20

PARTITIONING OF χ^2 FOR THE DATA OF TABLE 19

Source of Variation	D.F.	χ^2	*P*
Parallelism	1	0·61	0·5-0·3
Heterogeneity	4	3·85	0·5-0·3

TABLE 21

THE RATIO OF THE M.E.D.'S AND THE FIDUCIAL LIMITS OF ERROR ($P = 0.05$) OF THE RATIO FOR THE DATA OF TABLE 19

Treatment	Log_2 M.E.D.	S.E. of Log_2 M.E.D.	Ratio M.E.D. of Untreated to M.E.D. of Treated	Fiducial Limits of Error ($P=0\cdot05$) of Ratio
Untreated ..	1·914	0·384	1·01	0·39-2·64
I^{131}	1·893	0·592		

observed. Also Miwa, Ito, and Hayazu (1940) demonstrated the enhancement of activity obtained with heart muscle. Recently the effect of incubating oestrone with blood has been examined. Two groups of workers have examined the problem and have obtained opposite results. Werthessen, Baker, and Borci (1948) found that blood inactivated oestrone whereas Bischoff, Katherman, and Yee (1951) have observed increased activity. The latter group have found that blood has no influence on oestradiol-3,17β and oestriol. The evaluation of the bioassay and extraction procedures used by these two groups is difficult if not impossible. However, there is good reason to suppose that oestrone is in some way modified in various tissues of the body, and this idea is further supported by the recent observations of Claringbold (unpublished data) that cyanide significantly increases the intravaginal activity of oestrone but has no effect on the intravaginal activity of oestradiol-3,17β.

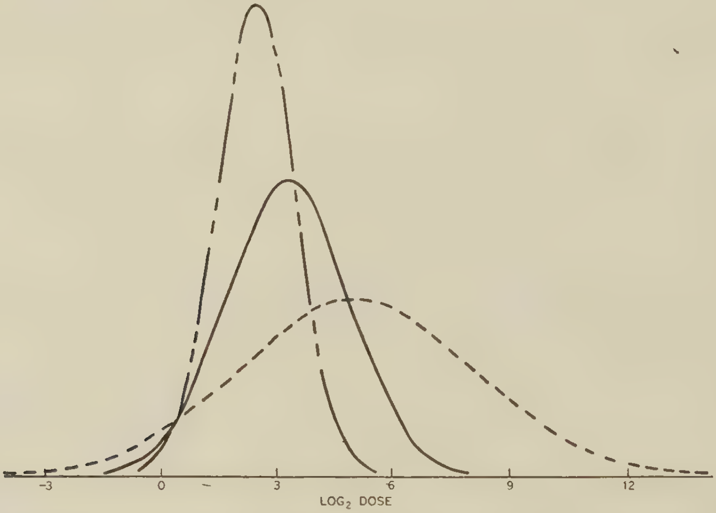

Fig. 4.—Theoretical normal distributions of threshold doses of oestrogen required to produce cornification calculated from the intravaginal dose response lines shown in Figure 1. —— - - —— Hypothyroidism; ———— normal; - - - - - hyperthyroidism.

The various metabolic interconversions of oestrogens have been discussed by Heard (1949). The known interconversions of oestrone, oestradiol-3,17β, and oestriol can be indicated as follows, whence it is seen that oestriol has not so far been shown to change to either of the others:

$$\begin{tikzcd}
\text{Oestrone} \arrow[rr, leftarrow, shift left] \arrow[rr, shift right] \arrow[dr] & & \text{Oestradiol-3,17}\beta \arrow[dl] \\
& \text{Oestriol}. &
\end{tikzcd}$$

Bischoff, Katherman, and Yee (1951) have some evidence that in blood at least some of the oestrone is converted to oestradiol-3,17β. It is possible that following absorption into the vaginal cells a similar process may occur. The depression of slope and the increased M.E.D. observed with oestrone when thyroxine is also administered may be due to interference with this process. The slope and M.E.D. with oestradiol-3,17β are unaffected; furthermore, this steroid is more active than oestrone. These facts may indicate that oestrone acts on the vagina only following conversion into oestradiol-3,17β, but this can only be resolved by further work.

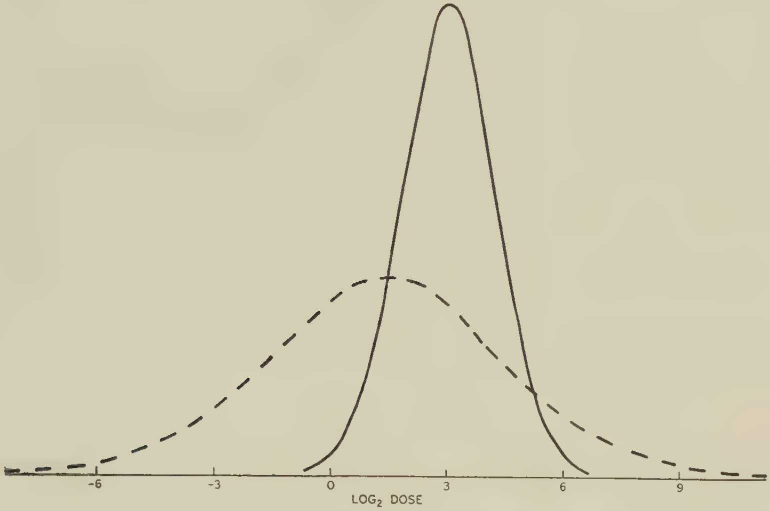

Fig. 5.—Theoretical normal distributions of the threshold doses of oestrogen required to produce cornification calculated from the dose response lines of Figure 3. ------ Oestradiol-3,17β; ———— oestrone.

The findings reported in this paper raise once more the problem of the relationship between the structure and function of oestrogens. The multiplicity of oestrogenic substances has interested people for many years but no theory has been put forward as to what makes a substance oestrogenic. The problem became of special interest following the discovery of synthetic oestrogens (see Dodds *et al.* 1939). For many years attempts have been made to find some steric configuration common to oestrogenic substances and a recent symposium on this subject (Gordon 1950) illustrates the lack of success in this respect. The failure has in part been due to the use of inaccurate and poorly understood bioassay procedures. The discovery of *pro-oestrogens,* as against *true oestrogens* (Emmens 1941, 1942), demonstrated an important group of oestrogenic substances possessing a structure which, in the body, is capable of transformation into a true oestrogen. True oestrogens, only, are capable of acting locally in small concentrations on vaginal cells and stimulating a characteristic biological response. It seems of paramount importance, therefore, that any oestrogenic substance be initially classified into either a pro-oestrogen or a true oestrogen.

As yet the relative activities of all true oestrogens administered intravaginally have not been determined. Those that have been determined indicate that the relative potencies are very similar (see Emmens 1950*b*), and on this basis it might be supposed that some steric molecular configuration is common to all true oestrogens. Two groups of observations suggest that this is not so:

(1) The finding by all workers using the intravaginal technique that oestradiol-3,17β is the most active compound in 50 per cent. aqueous glycerol (Muhlbock 1940; Emmens 1950*a*), in 1 per cent. egg albumin (Biggers 1951*a*), and in distilled water, as used in the present thiouracil experiments; and

(2) The differential action of thiouracil and cyanide on the oestrone and oestradiol-3,17β dose response lines.

With these results in mind it is tempting to suggest that the property common to all true oestrogens is a molecular structure which can be modified by local metabolic processes in the vaginal cells to give a compound which can initiate the biological response. A large amount of work remains to be done to ascertain the truth of this hypothesis.

The results obtained with thyroxine, methyl-thiouracil, and ^{131}I are interesting from the point of view of thyroid physiology. They provide an example of a peripheral interaction of the ovarian and thyroid hormones. The results emphasize that it is important to specify the steroid hormone being studied and that it is hazardous to generalize from the results obtained with one oestrogen.

V. ACKNOWLEDGMENTS

We wish to express our thanks to Professor C. W. Emmens for his advice and criticism during the course of this work. We are also indebted to Mr. T. R. Bradley for his aid in studies on the effect of methyl-thiouracil on the strain of mice employed.

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THE DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS IN FERTILIZATION AND EARLY SEGMENTATION

I. STUDIES ON LIVING EGGS BY ULTRAVIOLET MICROSCOPY

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[*Manuscript received December* 9, 1952]

Summary

Observations have been made on the absorption of ultraviolet radiation at 260 mμ by living rat eggs, as full-grown oocytes and during fertilization and early cleavage. Precautions have been taken to permit the results to be interpreted in terms of nucleic acid concentrations.

High concentrations of nucleic acid have been observed around the nucleolus of the oocyte, in the sperm head during its metamorphosis in the egg, in the nucleoplasm of the early pronuclei, and in the nuclei of segmenting eggs, particularly in the region immediately about the nucleoli. Little or no nucleic acid was detected within the nucleoli at any stage.

A moderate concentration of nucleic acid has been observed in the cytoplasm of the oocyte and of the egg during fertilization; in the course of early cleavage the concentration decreases progressively. There appears to be some association between nucleic acid and the granular elements of the cytoplasm.

Quantitative data are presented on the nuclear and nucleolar volumes, and nucleolar surface areas, in living 2-cell, 4-cell, and 8-cell eggs. In general these functions decrease in the proportions 4:2:1 with successive segmentations.

The observations made are considered to provide general support for the following concept: The egg contains a store of nucleic acid in its cytoplasm, and certain specifically limited amounts of nucleic acid, contributed by the sperm head and the egg chromosomes, are contained within the pronuclei. During the pronuclear phase the function of nucleic acids is obscure, but in the early segmentation nuclei a structure disposed about the nucleolus, and containing a high concentration of nucleic acid, is progressively developed at the expense, in part, of the cytoplasmic store of nucleic acid. This structure may well be analogous to Caspersson's "nucleolus-associated chromatin" and be involved in protein synthesis during embryonic development.

I. INTRODUCTION

In previous communications the cytology of fertilization, as seen in the living egg of the rat by phase-contrast microscopy, has been described (Austin 1951, 1952). It was recognized, however, that though the phase-contrast microscope is unexcelled for the study of structure in living cells, no specific information of a chemical nature could be obtained by this means alone. For a better understanding of fertilization, some knowledge is needed of the chemical processes which accompany or underlie the visible structural changes.

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In recent years, much attention has been given to the nucleic acids and to the important role they appear to play in cell function (Brachet 1950; Caspersson 1950). Moreover, many of the cytochemical techniques employed may readily be applied to mammalian eggs. For these reasons investigations have now been made on the distribution of nucleic acids in the rat egg during fertilization and early segmentation. Two methods have been used: the observation of ultraviolet absorption at 260 mμ in living eggs, and the histological study of fixed eggs with the aid of the Feulgen reaction. These methods were selected because they involve well-established procedures and seemed the most likely to yield useful results. The use of ultraviolet microscopy for the study of living cells is examined by Ludford, Smiles, and Welch (1948), who also employed phase-contrast microscopy as a collateral method. In the present paper observations on the ultraviolet absorption of living eggs are described and discussed; a second paper will be devoted to results obtained by histological methods involving principally the Feulgen reaction.

Microscopic observations on the ultraviolet absorption of cells were probably made first by Köhler (1904), but the method has been developed and exploited most effectively by Caspersson and his associates, whose work is summarized in a recent book (Caspersson 1950). Most of the work has been done on cells which were actively growing or secreting, such as cells from embryonic, tumour, or glandular tissues. Little attention has been paid to the ultraviolet absorption in the eggs of any species; there appear to be only the reports of Caspersson and Schultz (1940) on *Psammechinus*, of Harvey and Lavin (1944) on *Arbacia*, and a preliminary note by Flax (1951) on the mouse.

The stages of fertilization which will be referred to in this paper are based upon phases of development of the male pronucleus and have been described in a previous communication (Austin 1952). Briefly, the sperm, shortly after entering the vitellus, undergoes changes which include the detachment of the head and the metamorphosis of this into a faintly seen "grey zone," from which is formed the male pronucleus. The development of the male pronucleus passes through six stages, designated as follows: 1 and 2, early and late primary growth, in which the nucleoli appear, enlarge, and coalesce; 3, the single nucleolus stage; 4 and 5, early and late secondary growth; and 6, full development. In the last three stages secondary nucleoli appear, grow, and gather near the middle of the pronucleus, while the large nucleolus formed at stage 3 dwindles in size. After maintaining their maximum size for a period, the male and female pronuclei diminish in size and finally disappear, giving place to the chromosomes of the first segmentation spindle.

II. METHODS

Adult female rats, killed at the appropriate times after mating, provided oocytes and also eggs in various stages of fertilization and early cleavage. The eggs were dissected from the ovary or from the fallopian tubes under normal saline solution and placed on a fused quartz slide. A coverslip of similar material, with a layer of vaseline applied to its edges, was placed over the preparation and pressed down until the internal structure of the eggs could be clearly seen under the microscope, i.e. until the vertical depth of the egg had been reduced to about 20 μ (Austin 1950). After a preliminary examination with the phase-contrast microscope a photograph was taken at a selected focal point. For this purpose the 4-mm. objective was used as it had about the same depth of focus as the objective of the ultraviolet microscope. The slide was transferred to the ultraviolet microscope and approximately the same focal level found in visible light. A second photograph was taken at the desired wavelength in the ultraviolet.

The optical parts of the ultraviolet microscope consisted of spherical reflecting condenser and objective, both of N.A. 0.78 and focal length 3 mm.* Wavelength was isolated by means of the monochrometer from a Beckman model DU quartz spectrophotometer; for a 2-mm. slit width the range in the region of 260 mμ is ± 2.5 mμ with this instrument. The source of radiation was the standard hydrogen lamp used with the Beckman spectrophotometer.

Photographs in the ultraviolet were taken on Kodak "Ortho-X" plates, at exposures of 6-10 min. Tests showed that living rat eggs could tolerate at least 20 min. exposure at 260 mμ without any observable change in structure or absorption characteristics. Magnification at the plate was × 330; further enlargement was obtained photographically.

III. OBSERVATIONS

(*a*) *Ultraviolet Absorption in the Full-Grown Oocyte*

In the ovarian egg, obtained shortly before the time at which the final maturation changes occur, the distribution of ultraviolet absorbing material is clearly defined (Plate 1, Figs. 1*a*, 1*b*). Strongest absorption occurs in the region immediately surrounding the nucleolus and in small irregular bodies which can be seen in the nucleus often in the vicinity of the nucleolus. The nucleolus itself appears to absorb slightly, but this effect may well be due to the shell of strongly absorbing material just described. The nucleoplasm shows little or no absorption of ultraviolet radiation. In the cytoplasm there is quite strong absorption and this is distributed evenly throughout the egg.

(*b*) *Ultraviolet Absorption during Fertilization*

The structure which is formed from the sperm head soon after it has entered the egg cytoplasm, and which has been termed the "grey zone," is seen to absorb radiation strongly (Plate 1, Figs. 2*a*, 2*b*), so that it can be distinguished from the adjacent group of cytoplasmic granules more easily in the ultraviolet than the phase-contrast picture. Plate 1, Figures 2*a* and 2*b*, also shows the group of chromosomes extruded in a polar body, and these have an even greater absorption of ultraviolet radiation. The polar body appears to lie within the egg in these pictures but has in reality been pressed into the upper surface of the egg by the overlying coverslip.

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* These parts were constructed by the Division of Physics, C.S.I.R.O., Sydney.

The early primary stage of growth of the male pronucleus is seen in Plate 1, Figures 3*a*, 3*b*, and 3*c*. When first observed, this egg showed a clearly defined "grey zone." Soon, early nucleoli began to appear (Fig. 3*a*) and, about 15 min. later a picture was taken at 260 mμ (Fig. 3*b*). Absorption of ultraviolet radiation is very distinct in the nucleoplasm, but there is an indication of weak absorption in the nucleoli. Pronuclear development continued, apparently quite unaffected by the ultraviolet irradiation, and, after a further 15 min., it reached the stage shown in Figure 3*c*. Another view of a male pronucleus in early primary growth, but seen "end-on," shows the strong ultraviolet absorption in the nucleoplasm and its relative absence from the nucleoli (Plate 2, Figs. 4*a*, 4*b*). In this picture the intensely absorbing mass of chromosomes which will shortly form the female pronucleus can also be seen. Plate 2, Figures 5*a* and 5*b*, shows a male pronucleus in the phase of late primary growth; absorption in the nucleoplasm is still evident but it is now appreciably weaker. At the single nucleolus stage (Plate 2, Figs. 6*a*, 6*b*) absorption has become vague in the male pronucleus although it is still quite distinct in the smaller female pronucleus. In subsequent pronuclear growth the absorption in the nucleoplasm of both pronuclei becomes so weak that it cannot be discerned with any certainty at the stages of late secondary growth and full development (Plate 2, Figs. 7*a*, 7*b*). The rings which can be seen outlining the nucleoli in the ultraviolet picture may well be due to refraction alone.

Throughout most of the fertilization stages there is a moderately strong, diffuse, cytoplasmic absorption. To a certain extent the absorption is associated with granular elements in the cytoplasm. This is particularly noticeable in the early stages of fertilization when the granular elements show some aggregation and are generally absent from the peripheral cytoplasm (Figs. 2 and 3). Nevertheless the cytoplasm, which is free of granules, also absorbs quite strongly.

(*c*) *Volume and Surface Area Changes in Early Segmentation*

To obtain information on the changes in nuclear and nucleolar volume, and in nucleolar surface area, during the process of early segmentation, measurements were made of the diameters of nuclei and nucleoli. The diameter of the nucleolus and the longest and shortest horizontal diameters of the nuclei were measured with an eyepiece micrometer. The vertical depth of the nucleus was estimated with the aid of the fine adjustment on the microscope.

When the nucleus re-forms, after cleavage, it is initially much smaller than a mature nucleus and contains a number of small, closely arranged, nucleoli. As the nucleus matures it enlarges, several of the nucleoli coalesce, and more free space appears within the nucleus. The attempt was made to select for measurement only those eggs in which the nuclei appeared to be mature. This, however, is open to subjective error and it is probable that some relatively immature nuclei have been included in the data at each of the segmentation stages.

From the data obtained, the volumes of nuclei and nucleoli, and the surface area of nucleoli, were calculated and are recorded in Table 1. Measurements were made on 40 2-cell, 20 4-cell, and 10 8-cell, eggs, i.e. on 80 nuclei for each stage of segmentation.

The number of nucleoli per nucleus decreases from a mean of 5.4 in the 2-cell egg, to 4.0 in the 4-cell, and 1.4 in the 8-cell egg. The total volumes of nucleoli per nucleus are reduced from a mean of 324 cu. μ in the 2-cell egg, to 159 cu. μ in the 4-cell, and 75 cu. μ in the 8-cell egg. Corresponding values for total nucleolar surface area were 379, 211, and 92 sq. μ respectively. The figures for both nucleolar volume and surface area are thus approximately halved at each segmentation. Nuclear volumes showed a reduction from a mean of 2808 cu. μ in the 2-cell egg to 1206 cu. μ in the 4-cell, and 840 cu. μ in the 8-cell egg. These figures differ from the expected ratio of 4:2:1; the mean volumes of the 4-cell nuclei are smaller than expected, and those of the 8-cell nuclei a little larger.

TABLE 1

QUANTITATIVE CHANGES IN THE NUCLEI AND NUCLEOLI OF LIVING RAT EGGS DURING THE FIRST THREE STAGES OF CLEAVAGE

	No. of Eggs	Volume of Nuclei		Number of Nucleoli per Nucleus		Total Volume of Nucleoli per Nucleus		Total Surface Area of Nucleoli per Nucleus	
		Range (cu. μ)	Mean (cu. μ)	Range	Mean	Range (cu. μ)	Mean (cu. μ)	Range (sq. μ)	Mean (sq. μ)
2-Cell eggs	40	1867-3630	2808 ±42*	2-11	5·4 ±0·2*	241-616	324 ±6·0*	236-509	376 ±6·9*
4-Cell eggs	20	611-1886	1206 ±32	2-9	4·0 ±0·15	110-253	159 ±3·1	149-376	211 ±4·2
8-Cell eggs	10	540-1298	840 ±16	1-3	1·4 ±0·07	51-106	75 ±1·5	74-133	92 ±1·5

* Standard error.

(*d*). *Ultraviolet Absorption during Early Segmentation*

The distribution of ultraviolet absorption showed distinct and progressive changes during the early segmentation of the egg. Cytoplasmic absorption, which was noted to be distinct in the fertilization stages, was of about the same intensity in the 2-cell egg (Plate 3, Figs. 8*a*, 8*b*). In the 4-cell egg (Plate 3, Figs. 9*a*, 9*b*) there was an appreciable reduction in the intensity of absorption in the cytoplasm except in areas near the nuclei where it was about the same as in the 2-cell egg. This change was even more evident in the 8-cell egg (Plate 3, Figs. 10*a*, 10*b*) and in the 16-cell egg (Plate 3, Fig. 11); in these the absorption in the outlying parts of the cytoplasm was much less than in the 2-cell egg. The absorption near the nuclei was associated with the granular cytoplasmic elements which were gathered there. The cytoplasmic granules show a distinct change in distribution during segmentation. In the 2-cell egg their arrangement is similar to that seen during the later part of fertilization. With succeeding stages of segmentation the cytoplasmic granules appear to undergo a steady reduction in number and tend to aggregate around the nuclei. Even where free of granules, however, the cytoplasm still shows definite absorption of radiation.

The most striking changes seen during segmentation occurred in the nuclei. In the 2-cell egg the rings about the nucleoli were darker than those seen in the later pronuclei. Peri-nucleolar absorption was more distinct in the 4-cell egg, and stronger still in the later segmentation stages. In these eggs the absorption about the nucleoli could be related to the presence of irregular peri-nucleolar masses which could be clearly discerned with the phase-contrast microscope (Plate 3, Figs. 12*a*, 12*b*). Throughout all these stages the nucleoli themselves showed little or no ultraviolet absorption.

IV. INTERPRETATION

The interpretation of photographs obtained in the ultraviolet must be made with care. In general, the dark regions of the print may be considered to represent areas of absorption, provided the loss of radiation through refraction, particularly by small particles, is distinguished from the loss due to absorption. After true absorption has been shown to occur, it is then necessary to demonstrate that the absorption can be imputed to nucleic acids. Although full proof of this does not appear to be possible yet (Danielli 1947), good evidence for the presence of nucleic acids can be obtained by observing the absorption at 280 mμ and 300 mμ as well as at 260 mμ. From the evidence available (Pollister and Ris 1947; Caspersson 1950; Leuchtenberger *et al.* 1952), it can be concluded that, if the absorption is higher at 260 mμ than at 280 mμ, and is negligible at 300 mμ, nucleic acids are chiefly responsible.

To establish these points for rat eggs in the present study, the 8-cell egg shown in Plate 3, Figures 10*a* and 10*b*, was also photographed at 280 mμ and 300 mμ (Plate 3, Figs. 10*c* and 10*d*). It may be seen that most of the image obtained at 260 mμ and 280 mμ is caused by the absorption of radiation, for the image at 300 mμ is very much fainter. Indeed, the loss of radiation due to refraction is only appreciable in some of the granular elements in the cytoplasm, and, to a smaller degree, as a faint ring about each nucleolus, and just within the zona pellucida. It is, of course, possible that some of the cytoplasmic granules absorb specifically at about 300 mμ, but for the purposes of this investigation the picture obtained at 300 mμ is taken to represent the effects of refraction.

Comparison between the absorption evident at 260 mμ and at 280 mμ (Plate 3, Figs. 10*b*, 10*c*) shows that it is distinctly stronger at 260 mμ in the nucleus, particularly in the peri-nucleolar material, and somewhat stronger in the cytoplasm. The absorption in these regions may therefore be imputed principally to the nucleic acids.

Observations by Brumberg and Larionow (1946) suggest that nucleic acids may show little or no absorption of ultraviolet in living cells: that the cells must be killed before the characteristic absorption is displayed. In the present investigations, however, absorption is found in cells which are manifestly still alive. It has already been mentioned that the eggs show no change in structure or in absorption characteristics even after irradiation for two or three times the period required for photography. Furthermore, pronuclear development in the egg shown in Plate 1, Figure 3*a*, continued in the normal manner, as illustrated in Plate 1, Figure 3*c*, even after the photograph of its ultraviolet absorption (Plate 1, Fig. 3*b*) had been taken.

V. Discussion

From the premises stated in the previous section, it seems sound to conclude that nucleic acids exist in the living, segmenting rat egg in concentrations which are approximately represented by the strength of absorption at 260 mμ. In the early stages of fertilization the nucleic acids of the sperm head may reasonably be supposed to enter into the composition of the "grey zone" and early male pronucleus, and to be responsible in large measure for the strong ultraviolet absorption noted in these structures. Similarly, the nucleic acids of the egg chromosomes may be held largely responsible for the strong absorption shown by the early female pronucleus.

During the early growth of the pronuclei the nucleic acid concentration of the nucleoplasm falls rapidly, so that, after the single nucleolus stage, detection of nucleic acid through ultraviolet absorption becomes doubtful. It can be calculated from data previously described (Austin 1952) that by the time the single nucleolus stage has been reached in the living rat egg, the nucleoplasm of both pronuclei has undergone an increase in volume of about five times, so that the drop in nucleic acid concentration may be regarded as a consequence of dilution alone. In the remaining stages of pronuclear development dilution would continue, since the volume of the nucleoplasm increases by a further five times approximately. The falling concentration with increasing nucleoplasmic volume is consistent with the idea that the amount of nucleic acid originally contributed to each pronucleus remains unchanged during pronuclear development.

The rings seen about the nucleoli of the late pronuclei may connote a peri-nucleolar distribution of nucleic acid, as in the oocyte and segmenting eggs, but this is hypothetical as the rings are faint and may well be due to the effects of refraction alone. It has been suggested that a function of the pronuclei may be some form of synthetic activity, such as the production of templates for future embryonic growth (Austin 1951). This may still be true, but evidently a protein synthesizing system such as that described by Caspersson and his associates is not involved, for the required high concentrations of nucleic acid about the nucleoli and pronuclei are lacking.

In the course of the early cleavage stages, the highest concentrations of nucleic acid in the living egg are found in the region immediately surrounding the nucleoli. This peri-nucleolar material is fairly certainly present in the 2-cell egg, but becomes progressively more evident in the 4-, 8-, and 16-cell stages, and assumes various irregular shapes in the process. The development of the peri-nucleolar structure is clearly related to changes in the surface area of the nucleoli. In the 2-cell egg the total nucleolar surface area is about 379 sq. μ, compared with values of about 730 and 345 sq. μ reported (Austin 1952) for the fully formed male and female pronuclei respectively. During the early segmentation stages, the nucleolar surface area is approximately halved at each division. Changes of this order are sufficient to account for a large part of the apparent increase in the mass of the structure formed by the peri-nucleolar material.

If, as seems likely, the peri-nucleolar material has a high content of desoxyribonucleic acid (DNA), it may be regarded an analogous to Caspersson's "nucleolus-associated chromatin" (Austin 1953). The system as conceived by Caspersson is, however, incompletely represented in the segmenting egg, for the nucleoli contain little or no nucleic acid at any of the stages investigated, and the nucleic acid concentration in the cytoplasm is decreasing rather than increasing. It is therefore suggested that the peri-nucleolar structure, which is built up during the first few cleavage divisions, is an early form of the machinery involved in active protein synthesis later in embryonic growth.

The observation of increasing nucleic acid concentrations in the nuclei during cleavage is in general agreement both with the conclusions of Brachet (1950) on sea-urchin eggs, and with those of Alfert (1950) on mouse eggs, and Lison and Pasteels (1950) on sea-urchin eggs. These authors all studied the distribution of DNA in fixed eggs, but whereas Brachet described increasing *amounts* of DNA in cleavage nuclei, Alfert, and Lison and Pasteels considered that the amounts remained about the same, so that only the *concentrations* increased. On the other hand the present observations do not support Hertwig's (1939) theory that the amount of chromatin in each nucleus is halved at each division in early cleavage.

The cytoplasm of the full-grown oocyte and of the egg during fertilization shows evidence of a moderately high concentration of nucleic acid. The distribution throughout the egg is even, except that there is an indication of slightly higher concentrations associated with the groups of cytoplasmic granules. During early segmentation the concentration of nucleic acid falls off progressively; there is little observable change in the 2-cell egg, but distinct reductions in later stages. Here again, cytoplasmic nucleic acid shows a tendency to be associated with the granular elements. Brachet (1937, 1945) proposed that the increase in nuclear DNA during segmentation occurred at the expense of cytoplasmic ribonucleic acid (RNA), and the observations just described are therefore in agreement with this theory. Some agreement is also found with Dalcq (1951) who noted an association of RNA with granular elements in the cytoplasm of the rat egg. On the other hand, Harvey and Lavin (1944) found that, in *Arbacia,* ultraviolet absorption was chiefly evident in the hyaline part of the cytoplasm.

The general conclusions drawn from the present study on the ultraviolet absorption of living rat eggs may be stated as follows: A store of nucleic acid exists in the cytoplasm of the oocyte and the unfertilized egg, and limited contributions of nucleic acid, made by the sperm head and the egg chromosomes, are later contained within the pronuclei. During pronuclear development the function of the nucleic acids is obscure, for the cytoplasmic form shows little change in concentration and the nuclear moiety seems merely to undergo a progressive dilution with pronuclear growth. In early segmentation more distinct changes occur; in the nucleus a peri-nucleolar structure is gradually developed, which contains a high concentration of nucleic acid and is probably analogous to the "nucleolus-associated chromatin" of Caspersson and his associates. This structure may well be involved in vigorous protein synthesis later in embryonic growth, but does not appear to be active during early cleavage. The additional nucleic acid required for the formation of peri-nucleolar material may be drawn from the cytoplasmic store, which clearly diminishes during early cleavage.

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EXPLANATION OF PLATES 1-3

PLATE 1

Stages of fertilization

Each egg was photographed in visible light with phase contrast (*a*), and in the ultraviolet at 260 mμ (*b*). × 1000.

Fig. 1.—A fully grown oocyte, showing strong ultraviolet absorption immediately around the nucleolus, and moderate absorption in the cytoplasm.

Fig. 2.—Strong absorption is evident in the "grey zone" (g.z.) and in the group of chromosomes (chr.) in the polar body.

Fig. 3.—The early primary stage of development of the male pronucleus. There was an interval of about 15 min. between each picture. In this egg and that shown in Figure 2 the absorption associated with the cytoplasmic granules can be seen.

PLATE 2

Stages of fertilization (*continued*)

Conditions were the same as for Plate 1.

Fig. 4.—Another early primary male pronucleus, this time disposed "end-on" to the observer. Strong absorption is apparent in the nucleoplasm and also in the group of chromosomes (chr.) from which the female pronucleus is to be formed. The nucleoli are relatively free of absorbing material.

Fig. 5.—A male pronucleus in the stage of late primary growth. Ultraviolet absorption in the nucleoplasm is weaker than in the early primary stage of development.

Fig. 6.—Male and female pronuclei at the single nucleolus stage. Nucleoplasmic absorption is now not very distinct in the male pronucleus but is still quite strong in the smaller female pronucleus.

Fig. 7.—Male and female pronuclei at the stage of full development. The nucleoplasm does not appear to absorb the ultraviolet radiation. The rings outlining the nucleoli may be due to refraction alone.

PLATE 3

Stages of early cleavage

Fig. 8.—Nuclei of 2-cell egg photographed with phase contrast (*a*) and in ultraviolet at 260 mμ (*b*). × 660. There are distinct rings of absorption around the nucleoli. Cytoplasmic absorption is still fairly uniform.

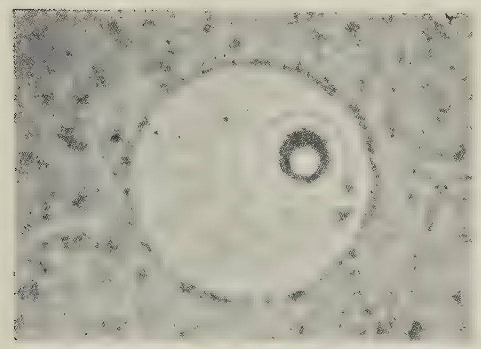
Fig. 9.—Two nuclei of a 4-cell egg photographed with phase contrast (*a*) and in ultraviolet at 260 mμ (*b*). × 660. Peri-nucleolar absorption is stronger than in the 2-cell egg, and the weaker absorption of the peripheral cytoplasm is more evident.

Fig. 10.—Three nuclei of an 8-cell egg photographed with phase contrast (*a*), and in ultraviolet at 260 mμ (*b*), 280 mμ (*c*), and 300 mμ (*d*). × 660. Absorption generally is stronger at 260 mμ than at 280 mμ, and particularly in the peri-nucleolar material. The picture taken at 300 mμ is considered to represent the effects of refraction alone. Compared with the 4-cell egg, peri-nucleolar absorption is stronger and absorption in the peripheral cytoplasm is weaker.

Fig. 11.—Portion of a 16-cell egg photographed at 260 mμ. × 660. Strong absorption can be seen in the nuclei and particularly about the nucleoli. The peripheral cytoplasm shows very weak absorption.

Fig. 12.—Nucleus of an 8-cell egg photographed with phase contrast (*a*) and in ultraviolet at 260 mμ (*b*). × 1300. The peri-nucleolar material is clearly seen and this has strong ultraviolet absorption whereas the nucleolus does not absorb the radiation appreciably.

DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. I



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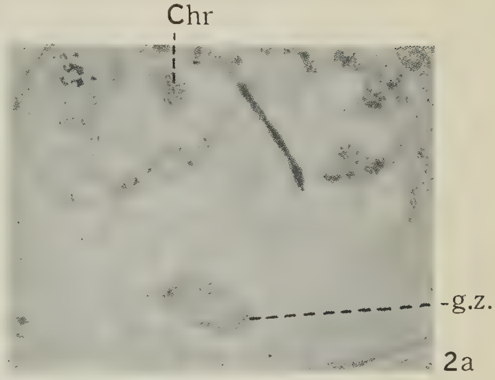

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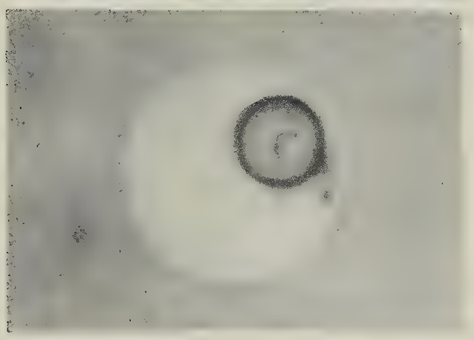

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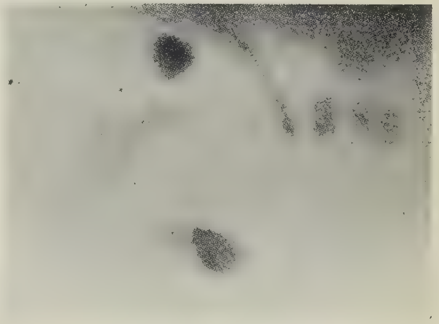

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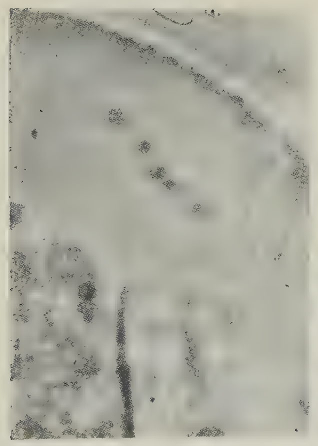

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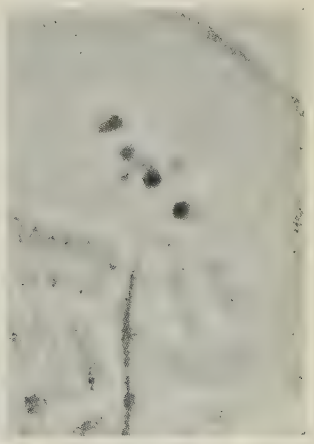

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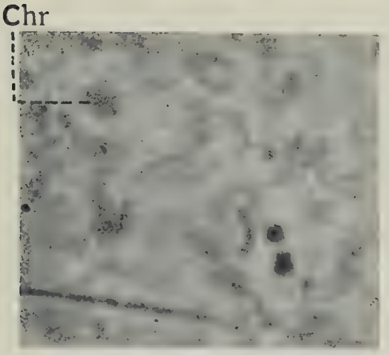
DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. I



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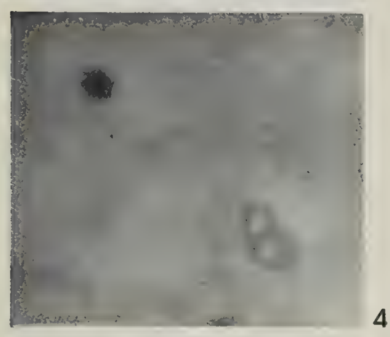

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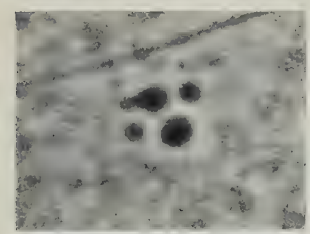

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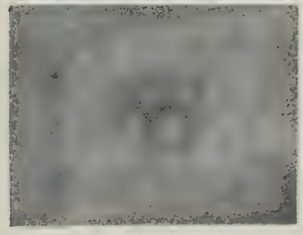

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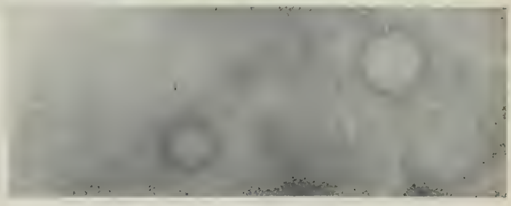

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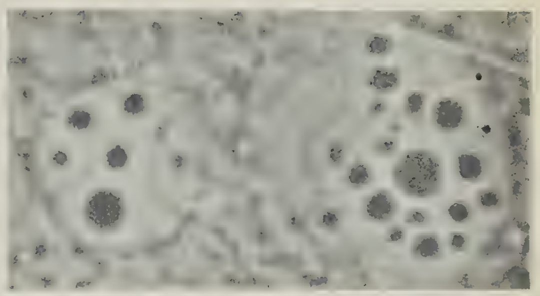

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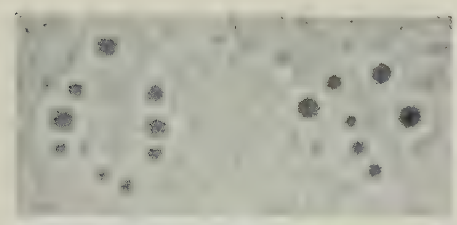
DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. I



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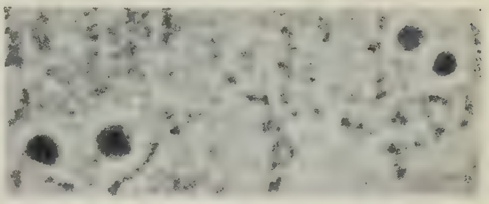

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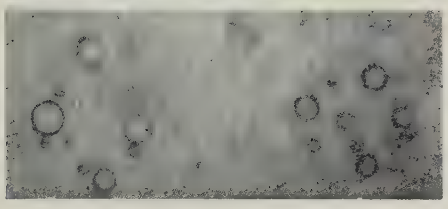

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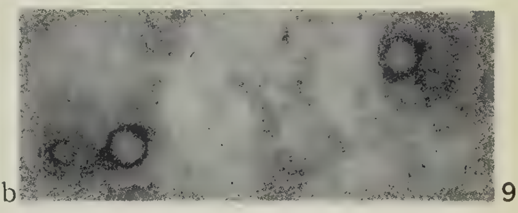

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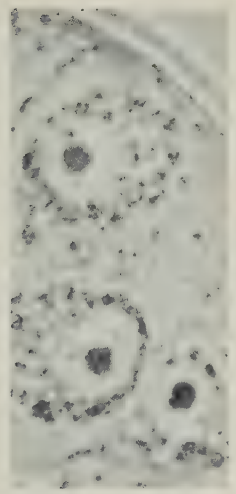

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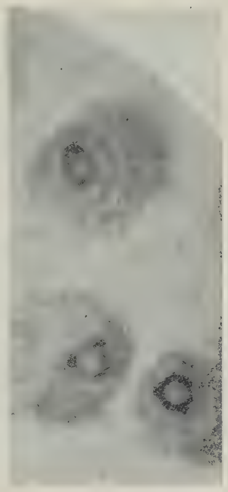

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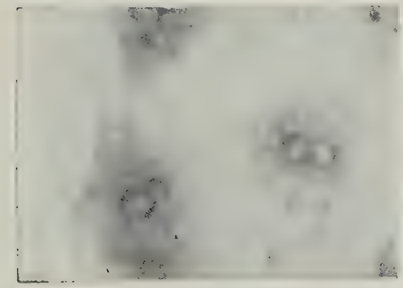

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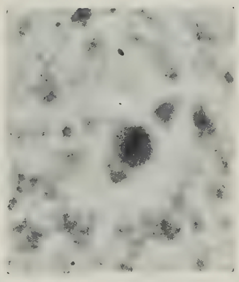

12a

12b

THE RESISTANCE OF TWELVE VARIETIES OF *TRITICUM VULGARE* TO *ERYSIPHE GRAMINIS TRITICI*

By A. T. Pugsley* and M. V. Carter†

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Summary

A genetic study has been made of the resistance of 12 varieties of *Triticum vulgare* Vill. to a South Australian isolate of *Erysiphe graminis tritici*. This collection has been resolved into two pathogenic components which may be differentiated by their reactions on the variety Chul.

The 12 varieties studied fall into three natural groups. Eight varieties of group *A* possess a common gene, Ml_t, which confers resistance to mildew at all stages of growth. Ml_t is completely linked with a gene conferring resistance to race 135AB of *Puccinia triticina* Erikss.

Group *B* is represented by a single variety which derives its mildew resistance from a second gene, Ml_u. This variety is susceptible to *P. triticina* 135AB.

The three varieties of group *C* are resistant to mildew from the three-leaf stage onwards and apparently owe their resistance to a third gene, Ml_s. These varieties are also susceptible to *P. triticina* 135AB.

I. Introduction

During the past quarter of a century, much evidence has been accumulated showing that a high degree of specialization occurs within many obligate pathogens of cereals and other cultivated plants. Only more recently have plant breeders become fully aware of the need for placing the emphasis upon gene-"race" relationships rather than upon large-scale "race"‡ surveys using differential varieties of unknown genetic constitution.

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‡ In plant pathological literature continual reference is made to "physiological races," a name used to designate isolates of pathogenic fungi, each possessing a characteristic range of pathogenicity on a set of so-called differential varieties. Such isolates have originally been made in the field, later to be maintained as clonal lines in a more or less static condition in the laboratory.

On the other hand, the use of the word *race* in genetics has a quite different connotation, being used always for a natural population ("Mendelian population" or "mating group") within which there is a continual exchange of genes during the sexual cycle.

Although physiological race, as a plant pathological term, has the official support of the Sixth International Botanical Congress, 1935, it is now suggested that, taking into consideration the developments in botanical science since that time, the term *race* be retained as a word expressing the genetical concept as outlined above.

If this be agreed upon, then some other term is needed for isolates of pathogenic fungi exhibiting characteristic parasitic qualities. Unfortunately the authors are unable to suggest an appropriate name so that in the present paper such isolates are designated as races.

The studies reported in this paper were undertaken with the object of identifying in 12 varieties of *Triticum vulgare* Vill. the genes for resistance to an isolate of *Erysiphe graminis tritici* occurring at the Waite Agricultural Research Institute, South Australia.

II. Review of Literature

Using Federation as the susceptible parent, Waterhouse (1930) showed that Thew possessed a single dominant gene for resistance to one of two races of *E. graminis tritici* occurring in the glass-house at Sydney University. He noted a linkage between this gene for mildew resistance and one for resistance to "Australian form 1" of *Puccinia triticina* Erikss. Nine other varieties (not specified) were found to possess this combined resistance to the above races of the respective pathogens.

Watson and Baker (1943) subsequently showed that the combined resistance of Thew was also a feature of the variety Kenya 744 and that it was due to a single dominant gene or to two completely linked genes.

Mains (1934) showed that Norka C.I.4377 possessed a single dominant gene for resistance to "U.S.A. form 1" of *E. graminis tritici* which was independent of a dominant gene for resistance to *P. triticina* "form 3." Red Fern was found to have a single dominant gene for resistance to "U.S.A. forms 1 and 2" of mildew, while Hope C.I.8178, which gave variable reactions to "form 1," apparently had a single recessive gene for resistance to this form. Sonora C.I.4293 likewise possessed a single recessive.

Pugsley (unpublished data) revealed the presence of one common gene in the varieties Thew, Kenya C6041 (Kenya 744), and Norka for resistance to the mildew population occurring at the Waite Institute. A second gene was detected in Sonora, and in each of the crosses between Sonora and the above-mentioned varieties, dihybrid ratios were secured in F_2 and F_3 populations.

Favret and Vallega (1949) ascribed the resistance of Axminster and Normandie to the Argentine races of *E. graminis tritici* as being due to a simple, almost completely dominant gene segregating independently of a gene governing resistance to *P. triticina* race 5, but linked to that for resistance to *P. triticina* race 20.

Sears (1950), by nullisomic analysis, has located a dominant gene for mildew resistance in Axminster on chromosone XI.

Lowther (personal communications 1951) has used the varieties Axminster C.I.8195, Ulka C.I.11478, Chul C.I.2227, Hope C.I.8178, and Normandie C.I.12747 as differentials to distinguish 10 physiologic races in U.S.A. From his data, it is evident that Axminster and Normandie differ in genetic constitution since the latter is resistant to a number of races to which the former is susceptible. Lowther reports Huron C.I.3315, Norka C.I.4377, and Converse C.I.4141 as reacting similarly to each of these 10 races; however, the latter finding is in disagreement with that of Taylor, Rodenhiser, and Bayles (1949) who reported that Converse was resistant to a mildew "collection" to which both Axminster and Norka were susceptible.

III. Materials and Methods

The following mildew-resistant varieties were used as parents in a series of crosses made during 1950 and 1951:

Thew	An Australian variety from ((Sinew × Improved Fife) × (Improved Fife × Hussar)) × Blount's Lambrigg selection.
Kenya C.6041 (N.S.W. Department of Agriculture)	Introduced from Kenya as B.F.4.2D.30.B.2 (L). Identical with Kenya 744 (Sydney University), and P.I.124737 (United States Department of Agriculture).
Axminster C.I.8195	Developed by Samuel Larcombe of Birtle, Manitoba, Canada, and believed to be a natural cross between Marquis and an unknown variety.
Converse C.I.4141	Origin not known.
Birdproof	Introduced by the N.S.W. Department of Agriculture from Basutoland, South Africa.
Huron C.I.3315	Derived from a cross made in 1888 between White Fife and Ladoga at the Central Experimental Farm, Ottawa, Canada.
Norka C.I.4377	Originated from a pure-line selection of common wheat separated from a plot of Kubanka durum wheat in 1908 at Akron, Canada.
Normandie	Derived from a cross Vilmorin 27 × Hybride 40 made in 1935. Leblond Villegats (Euré.) in France.
Ulka C.I.11478	Obtained by the Dickinson, North Dakota, U.S.A., Station, from a farmer who introduced it from Russia.
Sonora C.I.3036	Brought to the United States from Magdalena Mission, Northern Sonora, Mexico.
Indian C.I.4489	Origin not definitely known. Probably the result of a natural hybrid between Sonora and some other variety. Mildew-resistant selection made at the Waite Institute.
Sturgeon C.I.11703	Produced by the Wisconsin Agricultural Experimental Station, U.S.A., from a cross between Progress and Marquis.

The Australian variety Federation was used as the only susceptible parent.

The original isolation of *E. graminis tritici* was made from the variety Federation at the Waite Institute in 1940, and since then it has been maintained on that variety. Over the years 1940-51, this isolate gave consistent reactions on Thew, Kenya C.6041, Norka, Sonora, and Federation, and there was no evidence to indicate that it was other than a single physiologic race. During the winter months of 1951, Chul C.I.2227 was tested on several occasions, and gave a susceptible reaction. In April 1952, however, the anomalous behaviour of Chul during routine tests at the Waite Institute led to the separation of two races from the mildew population. At that time Chul gave a resistant reaction after the normal incubation period of 8-10 days; but pustules of mildew began to appear after 3 wk of exposure to infection from Federation plants growing alongside. Apparently the mildew on Federation at that time was composed of two races—one capable of attacking Chul and the second incapable of attacking that variety. These races have been designated *P* and *P*-1 respectively. The latter was freed from the original race *P* by three successive rapid transfers on Federation.

A culture of *P*-1 was taken to Canberra in May 1952, and tests in the glass-house during the winter of that year indicated that it remained free from race *P*. Reactions of the 12 resistant parental varieties to *P*-1 at Canberra were consistent with those obtained previously with both *P* and *P*-1 at the Waite Institute.

The susceptibility tests were carried out with seedling plants grown in the glass-houses of the Waite Institute, Adelaide, and the Division of Plant Industry, C.S.I.R.O., Canberra. Seedlings were grown in seed boxes and inoculated at the one- to two-leaf stage by dusting with spores from heavily infected plants of Federation. Several seedlings of the parental varieties and of Federation were included in each test. By this method 100 per cent. infection of susceptible seedlings was readily obtained. All 1951 tests were conducted at Adelaide using race *P* (with possibly small amounts of *P*-1 present). In 1952, some tests were conducted at Adelaide in the presence of both races, and the tests at Canberra were carried out with *P*-1 alone. There was no evidence to indicate that any of the varieties used, or their hybrid progenies, reacted differentially towards the two races.

In order to determine which varieties possessed the combined resistance of Thew to leaf rust and mildew, the parental varieties were tested against *P. triticina* race 135AB (Waterhouse 1952). Inoculum for the Adelaide tests was isolated from the field at the Waite Institute in November 1950, while that for the Canberra tests originated from a culture supplied by Dr. I. A. Watson of the Sydney University. A race of leaf rust capable of attacking Thew (race 138BB, Waterhouse 1952) isolated from the field at the Waite Institute in 1951 was also studied. The two races of *P. triticina* were used in examining linkage relationships between mildew and leaf rust resistance in certain crosses as outlined in Sections IV (*b*) and (*c*).

Seedlings for rust and mildew susceptibility tests were grown in 6-in. pots. The rust inoculum was applied to the first leaf and the seedlings then held for 24 hr in a humid chamber. The mildew inoculum was applied 2 days later. There was no visible evidence of an interaction in the symptom expression of the two diseases.

IV. EXPERIMENTAL RESULTS

Varietal reactions to the two races of *E. graminis tritici* and to the two races of *P. triticina* are given in Table 1.

TABLE 1

REACTION OF WHEAT VARIETIES TO RACES *P* AND *P*-1 of *E. GRAMINIS TRITICI* AND TO RACES 135AB AND 138BB OF *P. TRITICINA*

R=resistant; S=susceptible

Variety	Reaction to *E. graminis tritici*		Reaction to *P. triticina*	
	Race *P*	Race *P*-1	Race 135AB	Race 138BB
Group A				
Axminster	R	R	R	S
Birdproof	R	R	R	S
Converse	R	R	R	S
Huron	R	R	R	S
Kenya C6041	R	R	R	S
Norka	R	R	R	R
Normandie	R	R	R	S
Thew	R	R	R	S
Group *B*				
Ulka	R	R	S	S
Group *C*				
Indian	R*	R*	S	S
Sonora	R*	R*	S	S
Sturgeon	R*	R*	S	S
Federation	S	S	S	S
Chul	S	R	—	—

* These varieties characteristically developed some mildew if exposed to infection at the single-leaf stage, but were more resistant if grown to the three-leaf stage before inoculation. Sturgeon usually developed small lesions on the leaf sheath.

(*a*) *Inheritance of Mildew Resistance*

The resistant varieties have been classified into three groups (Table 1) of which group *A* comprises varieties resistant to mildew at all stages of growth and to *P. triticina* 135AB, group *B* (one variety) is resistant to mildew at all stages of growth but susceptible to *P. triticina* 135AB, and group *C* is resistant to mildew from the three-leaf stage and susceptible to *P. triticina* 135AB. The inheritance studies are discussed for each group in turn and for intra- and intergroup crosses. Results are summarized in Table 2.

(i) *Group* A *Crosses with Federation and Intragroup Crosses*.—This group comprises Axminster, Birdproof, Converse, Huron, Kenya, Norka, Normandie, and Thew.

The F_1 plants from crosses between these varieties and Federation were mostly grown in the field at the Waite Institute, a few being grown in the glasshouse during the winter. The absence of mildew infection in all cases indicated the complete dominance of resistance.

Table 2

CLASSIFICATION OF F_2 SEGREGATES FOR THE CROSSES INDICATED WITH RESPECT TO MILDEW REACTIONS

For parental reactions, see Table 1. For each cross: 1st line, 1951 Adelaide; 2nd line, 1952 Adelaide; 3rd line, 1952 Canberra. R = resistant; S – susceptible.

P value for all ratios exceeds 0.05

	Parent	Group *A*								Group *B*	Group *C*		
		Axminster	Birdproof	Converse	Huron	Kenya	Norka	Thew	Normandie	Ulka	Sonora	Sturgeon	Indian
	Federation	71R:23S 87R:23S 77R:34S	— 106R:32S —	530R:172S — —	71R:22S — 81R:29S	555R:183S 109R:38S —	520R:185S 135R:40S 286R:103S	200R:79S — —	— 333R:26S* 436R:26S	— 155R:54S* 186R:52S	— — 79R:34S	— — 86R:30S	308R:108S 59R:30S 89R:33S
Group *A*	Axminster		— — —	— — —	— — —	— — —	— — —	— 135R:0S 117R:0S	— — —	— — —	— — 109R:5S	— — —	— — —
	Birdproof			— — 114R:0S	— 128R:0S 229R:0S	— 121R:0S 119R:0S	— 109R:0S 120R:0S	— 109R:0S 231R:0S	— 106R:0S 241R:0S	— — —	— — —	— — —	— — —
	Converse				235R:0S — —	240R:0S — —	— 177R:0S 235R:0S	225R:0S — —	— 107R:0S 239R:0S	— — —	325R:81S† — —	88R:7S — —	354R:25S — —
	Huron					235R:0S — —	— 154R:0S 111R:0S	240R:0S — 109R:0S	— 118R:0S 237R:0S	— 253R:16S* 96R:3S	— 111R:8S* 222R:16S	303R:26S — —	227R:10S — —
	Kenya						— 82R:0S 115R:0S	203R:0S —	— 212R:0S 481R:0S	— 371R:16S* 333R:22S*	— 100R:6S* 228R:12S	— — —	— — —
	Norka							73R:0S — —	— 111R:0S 333R:0S	— — —	— — —	275R:53S† 82R:17S† —	216R:11S — —
	Thew								— — —	— 71R:3S* 90R:6S	150R:9S — —	— — —	467R:27S — —
	Normandie									— — 208R:0S	— — 185R:4S	— — 188R:4S	— 300R:8S* 234R:4S
Group *B*	Ulka										— 218R:19S 227R:12S	— — —	— — —
Group *C*	Sonora											— — 237R:0S	240R:0S — —
	Sturgeon												231R:0S — 233R:0S
	Indian												

For each cross:
first line = 1951 Adelaide
second line = 1952 Adelaide
third line = 1952 Canberra
R = resistant
S = susceptible

Expected Ratios

Group *A* (Normandie excepted) × Federation 3:1
Normandie × Federation 15:1
Group *B* × Federation 3:1
Group *C* × Federation 3:1
Group *A* (Normandie excepted) × Group *B* 15:1
Group *A* (Normandie excepted) × Group *C* 15:1

Normandie × Group *B* 63:1
Normandie × Group *C* 63:1
Group *B* × Group *C* 15:1
P value for all ratios exceeds 0.05

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* Resistant class includes some plants with slight infection of mildew.

† Expected ratios 13:3 rather than 15:1.

F_2 progenies were readily classified into resistant and susceptible classes which reacted like the respective parents in each case and, with the exception of Normandie, monohybrid ratios were obtained. There was no evidence of inconsistencies between tests using race *P* and race *P*-1.

In the cross Normandie × Federation, susceptible segregates constituted about 1/16 of the F_2 population, indicating the presence of two pairs of independently segregating genes. On one occasion an intermediate class was present (93 resistant, 14 intermediates, 7 susceptible), suggesting a 13:2:1 ratio rather than a 15:1, in which case it would appear that one of the two genes in Normandie is incompletely dominant for resistance.

Although undoubtedly Birdproof possesses the Thew gene, some evidence has been obtained suggesting the presence of additional genes in this variety. A further examination of Birdproof crosses is being made with a view to securing confirmatory evidence on this aspect.

With regard to intragroup crosses, in no case did susceptible segregates appear in the F_2, indicating that the eight varieties share a common gene. The reactions of these varieties to leaf rust support the view that this gene is identical with that originally studied in Thew by Waterhouse (1930).

(ii) *Group* B: *Ulka.*—F_1 plants of Ulka × Federation developed a trace of mildew infection at the Waite Institute when tested with race *P* although such heterozygotes were not always separable from the homozygous resistant class in F_2 progenies. At Canberra, F_1 plants were as resistant as the Ulka parent when tested with race *P*-1. The F_2 segregation in all cases was indicative of a monogenic difference.

(iii) *Group C Crosses with Federation and Intragroup Crosses.*—This group includes Indian, Sonora, and Sturgeon, the analysis of which proved more difficult. Because the parents exhibited a partial susceptibility when inoculated prior to the three-leaf stage, the seedlings were usually raised in a separate glass-house and inoculated at a later stage of development than was the case with crosses involving groups *A* and *B*. The F_1 hybrids between these varieties and Federation were intermediate in resistance between the respective parents, and classification of F_2 progenies was often difficult, due apparently to differences in expressivity of the heterozygote class. Intragroup crosses indicated the presence of a common gene in the three varieties, since no fully susceptible segregates were obtained. The F_3 data for Federation × Indian indicated a monogenic difference, and the hypothesis of a common major gene, incompletely dominant, is advanced for these three varieties.

(iv) *Intergroup Crosses.—Groups* A *and* B. Except for the special case Normandie, susceptible segregates appeared in F_2 progenies of these crosses, indicating the non-allelic nature of the Ulka gene with that of the Thew gene in the group *A* varieties. The data were in agreement with a 15:1 ratio.

No susceptible segregates appeared in a Normandie × Ulka F_2 population of 208 plants; however, a larger population would be necessary to differentiate between a 63:1 and a non-segregating population. At present, then, it is not possible to say whether the second gene of Normandie is identical with the Ulka gene.

Groups A *and* C. Again, susceptible segregates appeared consistently in F_2 progenies of all the crosses examined. On several occasions segregates with slight mildew development were detected and although these were usually classified with the resistant segregates, in some cases they were more readily included with the susceptible segregates, giving ratios approximating 13:3 rather than 15:1.

In each of the three crosses involving Normandie, the data were in agreement with a 63:1 ratio, indicating that each of the two genes in Normandie was different from the group *C* gene.

Groups B *and* C. Segregation of susceptible plants occurred in the only cross examined, namely, Ulka × Sonora, indicating the non-allelic nature of the Ulka gene with that of the Sonora gene.

TABLE 3

CLASSIFICATION OF F_2 SEGREGATES OF THE CROSSES NAMED WITH RESPECT TO MILDEW AND LEAF RUST 135AB REACTIONS

For parental reactions, see Table 1

Cross	Mildew Resistant		Mildew Susceptible		Ratio	Value of *P* for Ratio Indicated
	Leaf Rust Resistant	Leaf Rust Susceptible	Leaf Rust Resistant	Leaf Rust Susceptible		
Federation × Axminster	148	0	0	57	3:1	>0·3
Federation × Converse	130	0	0	43	3:1	>0·99
Thew × Federation	112	0	0	47	3:1	>0·3
Federation × Huron	80	0	0	30	3:1	>0·5
Normandie × Federation	80	23	0	6	12:3:0:1	>0·8
Norka × Federation	85	0	15	9	12:0:3:1	>0·3
Indian × Converse	131	31	0	12	12:3:0:1	>0·9
Thew × Indian	123	36	0	12	12:3:0:1	>0·5
Sonora × Thew	126	24	0	9	12:3:0:1	>0·3
Thew × Huron	109	0	0	0		
Thew × Birdproof	111	0	0	0		
Huron × Birdproof	112	0	0	0		
Normandie × Norka	103	0	0	0		

(*b*) *Association of Mildew Resistance and Resistance to* P. triticina *Race* 135*AB*

Earlier reference has been made to the fact that all the mildew-resistant varieties of group *A* were resistant to *P. triticina* race 135AB. In view of the reported linkage between resistance to these pathogens in Thew and Kenya, one would expect a similar relationship to hold for the other varieties of this group. Evidence for such complete linkage with respect to the varieties Thew, Axminster, Converse, and Huron when crossed with Federation is presented in Table 3. In each instance, mildew-resistant segregates were resistant to leaf rust and mildew-susceptible segregates were susceptible to leaf rust, there being no recombination classes.

The results from other crosses also listed in Table 3 (Indian × Converse, Thew × Indian, and Sonora × Thew) clearly indicate the segregation of two genes for mildew resistance, one of which is linked to that conferring resistance to race 135AB of leaf rust.

Evidence of linkage in the varieties Norka and Normandie is also indicated but obscured by the segregation of additional genes. In the cross Norka × Federation, all mildew-resistant segregates were also resistant to leaf rust, but the data indicate that Norka possesses a second gene for leaf-rust resistance which is not associated with mildew resistance. With regard to Normandie, earlier results have indicated a segregation of additional genes for mildew resistance when this variety is crossed with Federation. That one of these genes is associated with leaf-rust resistance while the other is not, is clear from the data presented in Table 3.

TABLE 4

CLASSIFICATION OF F_2 SEGREGATES OF THE CROSS NORKA × FEDERATION WITH RESPECT TO MILDEW AND LEAF RUST 138BB REACTIONS

For parental reactions, see Table 1

Cross	Mildew Resistant		Mildew Susceptible		Ratio	Value of *P* for Ratio Indicated
	Leaf Rust Resistant	Leaf Rust Susceptible	Leaf Rust Resistant	Leaf Rust Susceptible		
Norka × Federation (Adelaide)	34	19	13	6	9:3:3:1	>0·2
Norka × Federation (Canberra)	75	40	36	11	9:3:3:1	>0·05

(*c*) *Independent Inheritance of Mildew Resistance and Resistance to* P. triticina *Race* 138*BB*

From the data presented in Tables 1 and 3 it is evident that Norka possesses leaf rust resistance additional to that found in the other varieties of Group *A*; this would also be concluded from the data of Mains (1934) who found independent inheritance of mildew resistance and leaf rust resistance in crosses between Norka and susceptible varieties. The data of Table 4, where race 138BB was used in the place of race 135AB of leaf rust, lend evidence in support of Mains's work and show that Norka carries a second gene for leaf rust resistance which is inherited independently of the Thew gene. This second gene gives resistance to both races 135AB and 138BB of leaf rust.

V. DISCUSSION AND CONCLUSIONS

As a result of the studies here reported, two physiological races have been isolated from the population of *E. graminis tritici* at the Waite Institute, South Australia. These races may be distinguished by their reactions on the variety

Chul C.I.2227, which is susceptible to the isolate designated race *P* and resistant to that designated race *P*-1. Since race *P*-1 has been studied at Canberra in the absence of race *P* and no results obtained which conflicted with the Adelaide results, it may be stated that the inheritance of resistance in the varieties studied follows a similar pattern with respect to both races.

Reviewing the results of the genetic analysis, it is concluded that 11 of the varieties differ from the susceptible Federation by one gene, while the 12th variety, Normandie, differs by two genes. The possibility that Birdproof possesses additional resistance has been recognized, but final conclusions with respect to this variety must await confirmatory evidence. Furthermore, a number of the varieties have been shown to contain a common gene for resistance.

Three distinct genes for mildew resistance have been identified, with a possible fourth gene occurring in Normandie. In assigning symbols to these genes, the system of nomenclature suggested by Briggs and Stanford (1938) in work on mildew resistance in barley has been adopted. The need for a standard system of genetic nomenclature and symbols for the wheat plant has long been recognized although as yet no general agreement has been reached. The present designations should, therefore, be regarded as tentative only.

Ml_t, Ml_u, and Ml_s are suggested as designators of the genes for resistance common to varieties of groups *A*, *B*, and *C* respectively, and are so named because they were first detected in the varieties Thew, Ulka, and Sonora, respectively. Until its identity can be established positively, the additional resistance of Normandie is symbolized by Ml_u.

The genetic constitution of the 12 resistant varieties and Federation may be written:

Group *A*			
Axminster	Ml_t	ml_u	ml_s
Birdproof	Ml_t	ml_u	ml_s
Converse	Ml_t	ml_u	ml_s
Huron	Ml_t	ml_u	ml_s
Kenya C. 6041	Ml_t	ml_u	ml_s
Thew	Ml_t	ml_u	ml_s
Norka	Ml_t	ml_u	ml_s
Normandie	Ml_t	Ml_u?	ml_s

Group *B*			
Ulka	ml_t	Ml_u	ml_s
Group *C*			
Indian	ml_t	ml_u	Ml_s
Sonora	ml_t	ml_u	Ml_s
Sturgeon	ml_t	ml_u	Ml_s
Federation	ml_t	ml_u	ml_s

Since the 12 mildew-resistant varieties fall into three groups with respect to the races studied, one would expect some degree of correlation in the behaviour of the varieties of each group towards other races of mildew. Evidence of such a correlation has been given by Lowther (personal communication, 1951) who reports that Norka, Converse, and Huron reacted to 10 physiologic races in a manner quite similar to Axminster. Normandie was resistant to all races to which the latter group were resistant, but it was also resistant to additional races, no doubt because of the presence of its second gene for resistance. In no case has Normandie been susceptible to races incapable of attacking Axminster.

Taylor, Rodenhiser, and Bayles (1949) found Indian and Sturgeon to react alike to three physiologic races, but with respect to group *A* varieties, their results are at variance with expectation, since Converse was resistant and Axminster and Norka susceptible to one of their three races.

Newton and Cherewick (1947) reported parallel reactions of Norka and Axminster to three Canadian races and, to the extent to which it was tested, Thew reacted similarly.

Certain anomalous results have been reported for Huron by Mains (1933), Vallega and Cenoz (1941), Newton and Cherewick (1947), and Taylor, Rodenhiser, and Bayles (1949), but these discrepancies are almost certainly caused by genetic differences between the strains of Huron used. Local experience confirms the work of Taylor, Rodenhiser, and Bayles (1949) in demonstrating clear differences in mildew reaction between Huron R.L. 20 (C.I. 12663) and Huron C.I.3315.

The results of inheritance studies are in close agreement with those reported by Waterhouse (1930), Mains (1934), Watson (1943), and Vallega and Favret (1950), so that it is now well established that the Thew gene for mildew resistance, as it exists in several varieties, is very closely, if not completely linked with a gene for resistance to certain races of *P. triticina*, and at the same time inherited independently of genes conferring resistance to other leaf rust races.

As with mildew, a similar correlation of behaviour towards other leaf rust races might also be expected with the varieties of group *A*. This expectation was realized in the present study with respect to races 135AB and 138BB, and is additionally supported by the results of Vallega (1944) who found Axminster, Normandie, and Thew to react in essentially the same way to six Argentine leaf rust races. However, Waterhouse (1952) describes Norka as susceptible and Thew as resistant to race 10A of *P. triticina*.

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TISSUE TENSIONS IN *BRYOPHYLLUM CALYCINUM* SALISBURY

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Summary

Turgor changes following the isolation of various tissue systems from internodes of *Bryophyllum calycinum* Salisbury plants have been examined and related to the new environmental conditions. The structural changes in cambium-phloem parenchyma strips have been analysed.

In tissue preparations the effect of auxins on subturgid cells seems to consist of the lowering of the equilibrium volume (maximal turgidity) in water. This effect is concomitant with an increase in permeability which is particularly noticeable in plasmolysis.

As most of the effects observed are almost instantaneous and reversible, "growth" (as defined as an irreversible increase in cell size) is probably not involved.

I. INTRODUCTION

The presence of tensions in plants can be inferred from certain nastic movements of organs and from sudden, often irreversible, reactions to moisture and touch; they may be illustrated by the changes in shape following the separation of plant tissues. These tensions can be considered in two classes: those arising from turgor differences (e.g. pulvinar tensions) and those dependent upon imbibitional or hygroscopic effects, as in various dehiscence mechanisms.‡

F. W. Went (1934) showed that certain tensions dependent on turgor changes and on growth were sensitive to auxin; and this may be the key to reaction in the pea test. Recent studies have stressed the role of auxin in the development and resolution of tissue tensions—in the torsions developed in the growing apex (Snow 1950) and in the mechanism of the pea test itself (Schneider 1942). Even though it is a convenient object for auxin determination, the pea epicotyl is made up of a complex of tissues and consequently presents a system very difficult to analyse. In this paper some tissue preparations from mature internodes of *Bryophyllum calycinum* Salisbury are discussed.

If we consider a plant organ in physiological equilibrium with its environment, the sum of tensions at any surface is zero; however, if a force is applied, or if the organ is cut up, there will be a readjustment of tensions and a resultant change in shape. This change will continue until equilibrium is resumed. The shape at equilibrium will depend upon the structure of the isolated tissues, their former physiological condition in the plant, and the nature of their new environ-

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‡ In Bünning's monograph (1948) the subject of the "paratonic" movements is adequately treated. For hygroscopic effects the classical paper of Steinbrinck (1906) is stimulating, see also Ziegenspeck (1943).

ment. For an analogy we may compare the effect of an increase in temperature upon a bimetallic strip with the effect of water on a thin strip of subturgid tissue consisting of two types of cells (or on a detached epidermis with its cuticle). In such a simplified tissue-tension system we may consider the two cell types as antagonists.

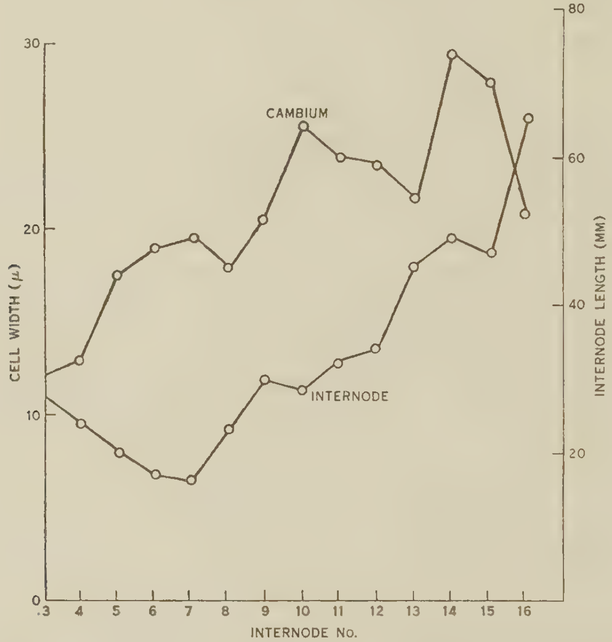

Fig. 1.—Width of the cambium cells and length of internodes. The numbers of the internodes on the abscissa refer to distance from apex.

Excised tissues of many plants show a distension of surface cells when put into culture on a moist medium. This hyperhydric increase in size has been linked with auxin content as well as the release of tensions (White 1951), nevertheless it is clear that a local release of tension may be followed by considerable modifications in the size and shape of cells, which, when irreversible, resemble growth. How much the balanced tensions contribute to the hydrostatic pressure in cells of an intact plant is not known—apart from the fact that they no doubt contribute to the effective wall pressure, and according to Broyer (1950) indirectly contribute to the "extrinsic turgor pressure."

An analysis of tissue-tension systems and their behaviour might yield some evidence as to the earlier phases in the action of auxins and also of the processes of morphogenesis and intracellular translocation of solutes in the intact plant. But there appears to be no necessity to go to such complex systems as those already in use for assaying growth substances; accordingly we have examined some simpler tissue tension systems in *Bryophyllum* and noted effects of indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). In particular we have been interested in the cambium-phloem system; and an attempt at analysis of its mechanism is presented.

II. Material and Methods

Bryophyllum calycinum Salisbury,* a perennial herb, is a member of the Crassulaceae; it is endemic in Africa but of almost cosmopolitan tropical and subtropical distribution. The stem has short internodes with two opposite leaves at each node. A central leaf-trace is flanked on each side by two and sometimes three subsidiary traces. Except in the upper internodes a closed woody cylinder surrounds a central medulla.

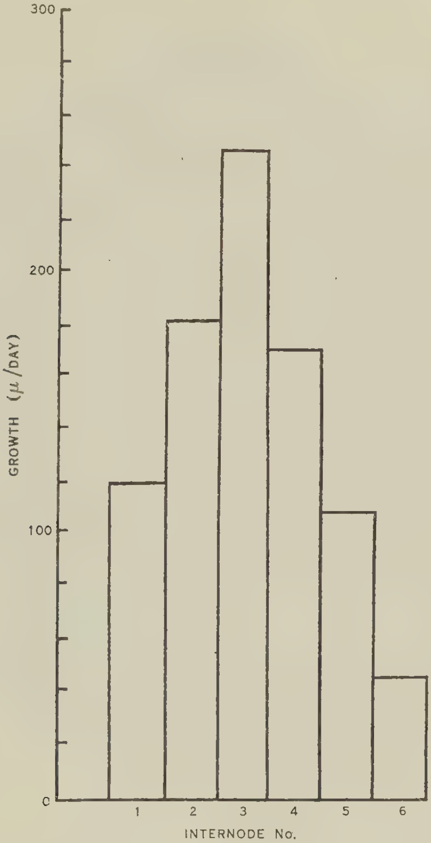

Fig. 2.—Distribution of growth (μ/day) over the stem of *Bryophyllum*. Average for four plants.

Longitudinal growth follows the general pattern of plants with well-defined internodes (van Burkom 1913). Internodal length reflects temperature conditions, and the stem with its varying internodes presents a rough record of the climate. A strong negative correlation was found between internodal length and the width of the cambium cells (Fig. 1). In short winter days the plant flowers and the new internodes grow slender and much longer than usual before terminating in an inflorescence.

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* Syn. *Kalanchoe pinnata* Persoon.

A batch of five 2-year-old plants was studied for evidence of maturation and new growth. A histogram (Fig. 2) shows the mean growth in μ/day (in winter). The full number of primary xylem bodies is usually formed by internode 5; cortical anthocyanin disappears at internode 7 where subepidermal cork formation is initiated. Secondary growth starts between the eighth and tenth internodes, and by the twelfth interfascicular xylem has completed the cylinder. There is considerable variation between individual plants, however.

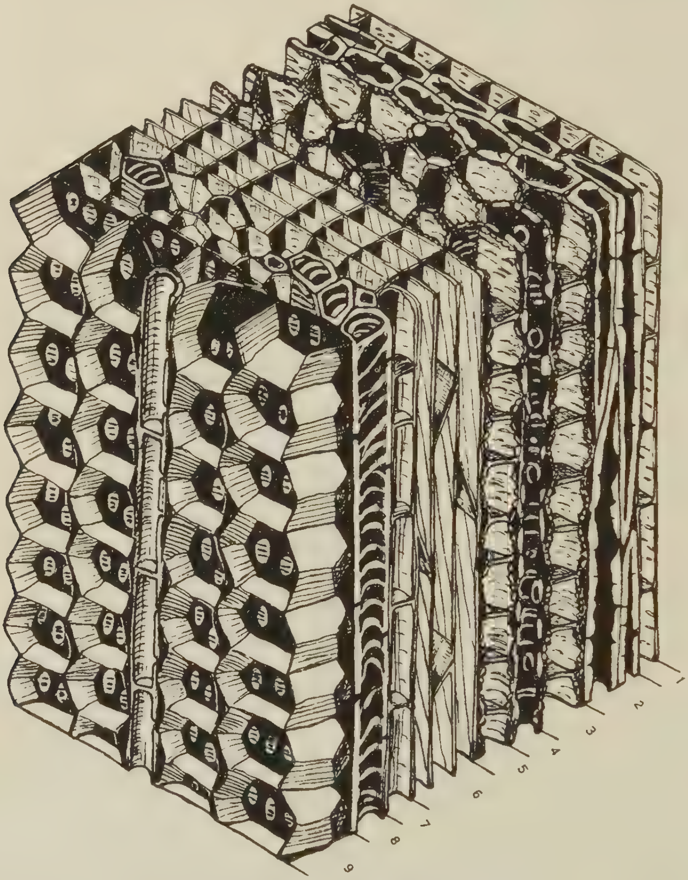

Fig. 3.—Diagrammatic representation of the tissues inside the endodermis. 1, Endodermis; 2, pericycle; 3, phloem parenchyma; 4, sieve-tubes; 5, phloem parenchyma; 6, cambium; 7, xylem parenchyma; 8, vessels; 9, central medulla. In the medulla, visible on the tangential plane, so-called tannin cells. Notice the "pit-field" on the tangential walls of the medullary parenchyma.

Figure 3 gives a diagrammatic representation of the tissues inside the cortex. The stem of *Bryophyllum* has three distinct pairs of antagonistic layers which may be isolated without much difficulty. Ability to remove the cambium and all outer tissues, which, below the first couple of internodes, yield easily from the xylem cylinder, was exploited by Loeb (1924) in his study of regeneration.[*] First an internode of suitable length is isolated and two parallel incisions are made along it; a piece separates easily with epidermis outside and cambium inside. Then with a new razor blade a sharp tangential cut is made between the cambium and the pericycle (i.e. about 2 mm from the inner surface), the small flap is gently pulled away with flat forceps or the fingers, and a clear strip is obtained consisting of cambium and phloem parenchyma. The ends are trimmed and this preparation is ready for use (Fig. 4). Microscopic examination, vital staining, and plasmolysis have rarely shown apparent damage to the cambium, while on the phloem side there are only a few torn cells. For physiological studies these strips are very useful, they are readily obtained, rapidly penetrated by solutions, and fairly simple in structure. From one internode 8-12 strips may be prepared.

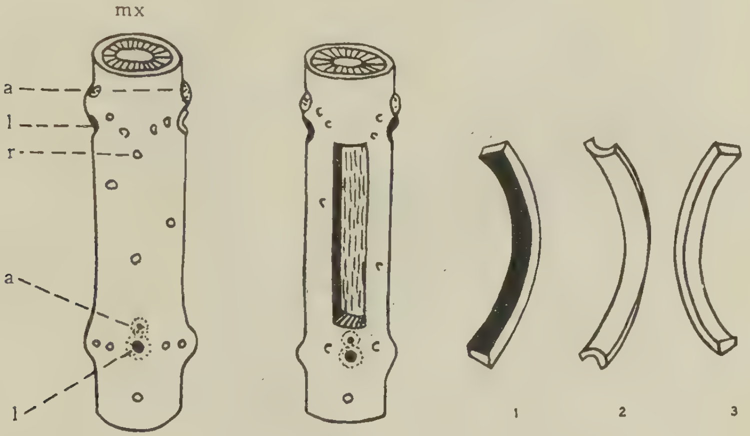

Fig. 4.—Internode of *Bryophyllum*. *a,* Axillary bud; *l,* leaf traces; *mx,* medulla-xylem; *r,* root primordia. 1, cortical strip removed from wood cylinder; 2, inner part of the strip (cambium plus phloem parenchyma); 3, outer part of strip (pericycle plus cortical parenchyma).

Other preparations are obtained by sectioning the xylem-medulla core, by taking transverse sections of the sheath removed from this core—"cortical rings"—or by pulling free the pericyclic and attached cortical tissue to form longitudinal strips as before.

Features of the cambium include the appearance of near-vertical striations (angle of 15-20°) in still-growing internodes, the presence of slightly wider cells opposite groups of primary xylem where interfascicular xylem has not yet formed, and the presence of air in the subcambial intercellularies; the air may find its way there during the preparation of the strip. It is important to use only cambium with conspicuously pointed cells; plants appear to vary in this regard.

Figure 5 shows a transverse section from the fourth internode of a plant with 24 groups of primary xylem indicating the regions where the preparations

[*] For other studies, also on hormonal action, in this plant, see Went (1930), Mrkos (1933), Uhrova (1934).

described are obtained. Prosenchyma present in this section includes spiral and ring tracheids and vessels in the xylem, the cambium, pericyclic sclerenchyma, tannin cells in the central medulla, and phloem sieve-tubes; while parenchyma is found in the central medulla, the collenchyma, and thin-walled cells in the xylem, phloem, and cortex. It is to be noted that in expansion and contraction the sieve-tubes behave like parenchyma. When tissues are isolated and placed in water it is possible to infer from the change in shape whether they were under compression or extension in the intact plant. Prosenchyma, with its longitudinal striations, is predisposed to changes in width, and is a logical antagonist for parenchyma, with its transverse striations, fitted for changes in length; however, lignified tissues like xylem, with no turgor reaction, cannot be considered this way. The stresses and strains in the tissues in *Bryophyllum* are depicted diagrammatically in Figure 6.

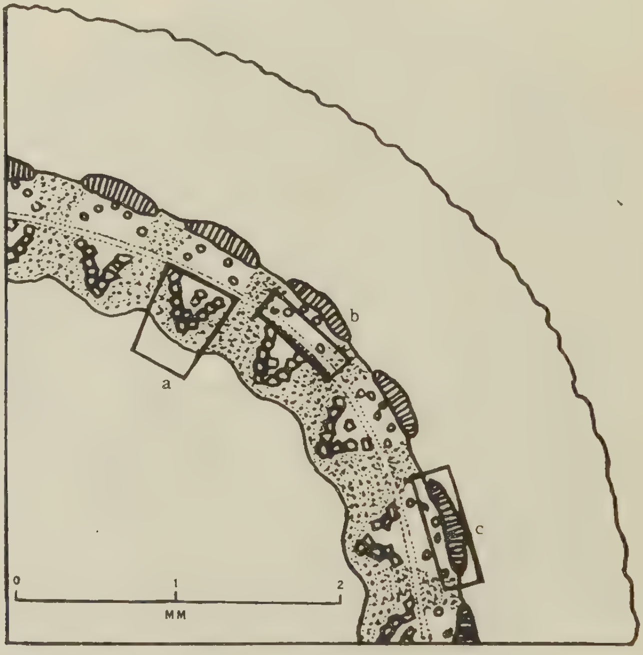

Fig. 5.—Part of transverse section through a young stem. *a*, Medulla-xylem strip; *b*, cambium-phloem strip; *c*, parenchyma-sclerenchyma strip. Fourth internode from the top.

Apart from differences in striation and turgor, the initial shape of a cell fits it for (and governs) any subsequent change in shape. We shall discuss briefly this difference with respect to two types of cell, both of prismatic shape, but with different outline in the tangential plane. A cambium cell and phloem parenchyma cell are shown in Figure 7. The tangential surface may be assumed to be proportional to the volumes; this is approximately so for the cambium, but for slight discrepancies in phloem parenchyma (Meeuwse 1941).

(*a*) *The Cambium Cell*

In transverse section the outline is rectangular: in the tangential plane the cells look like parallelopipeds, although pointed hexagons are often seen.* Let the sides of the parallelopiped be a and b, then the total length, L of the cell is

$$L = b + a \cos \theta,$$

where θ is the apical angle, and the width W is

$$W = a \sin \theta,$$

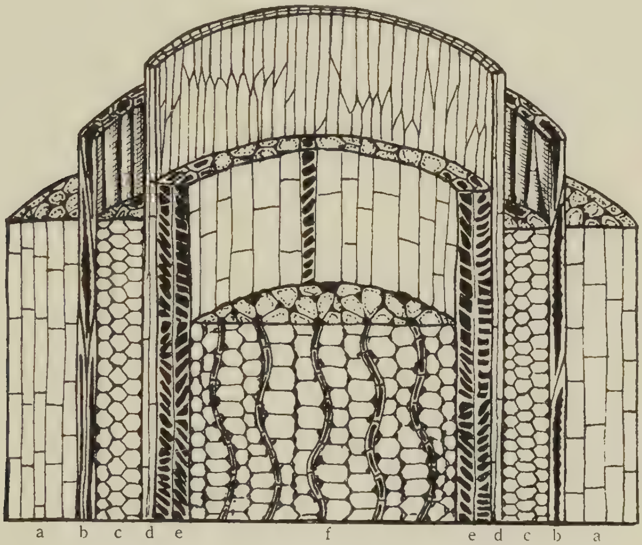

Fig. 6.—Distribution of tissue tensions in the stem (diagrammatic). *a,* Cortical parenchyma (slightly compressed); *b,* pericycle (under stress); *c,* phloem parenchyma (slightly compressed; *d,* cambium (stretched); *e,* xylem (slightly stretched); *f,* central medulla (with "tannin cells," much compressed).

from which it follows that

$$\left(\frac{L-b}{a}\right)^2 + \left(\frac{W}{a}\right)^2 = 1,$$

and it may be derived that the surface S in the tangential plane is

$$S = bW = ab \sin \theta,$$

which is maximal at

$$S = ab \text{ (rectangle).}$$

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* A similar system, the rhombic meshwork of cortical sclerenchyma in *Carica papaya* L., which is distended by secondary growth, was investigated by Arnold and Baas Becking (1949).

(*b*) *The Phloem Parenchyma Cell*

The parenchyma cell has transverse pits in the tangential and radial walls. The tangential aspect of a water-saturated cell is an equilateral hexagon, placed on its side. Again, from Figure 7, the length of the cell

$$L = 2p \sin \frac{\theta}{2}$$

and its width

$$W = p + 2p \cos \frac{\theta}{2},$$

from which it follows that

$$\left(\frac{L}{2p}\right)^2 + \left(\frac{W-p}{2p}\right)^2 = 1,$$

and the surface in the tangential plane is

$$S = \tfrac{1}{2}L(W+p) = 2p^2 \sin \frac{\theta}{2} + p \sin \theta,$$

maximal at

$$S = \frac{3}{2} p^2 \sqrt{3} \text{ (regular hexagon).}$$

The above only applies when the cell is approximately prismatic in shape.

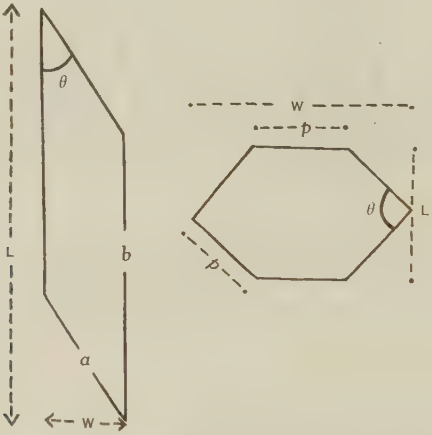

Fig. 7.—Schematical representation of cambium cell (left) and parenchyma cell (right).

Values of W and L for cambium and parenchyma cells are given in Table 1 and graphically represented in Figure 8.

It appears that in the cambium, an increase in width of the cell from 15 to 30 μ (100 per cent.) will cause a shortening from 197.6 to 193.3 μ—only 2.2 per cent. This means that, as a meshwork, the cambium, like the cortical sclerenchyma of *Carica*, may be distended or compressed a great deal without appreciable change in length. On the other hand, suppose a subturgid parenchyma cell to have a width of 30 μ and length 13.8 μ; these measurements will change on saturation (to a regular hexagonal outline) to 23 and 19.6 μ, changes of 24 and 70 per cent. respectively.

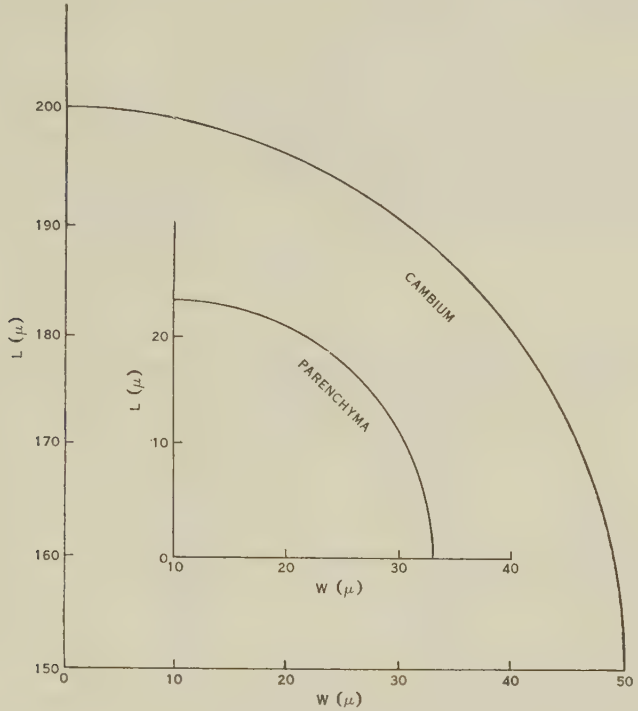

Fig. 8.—Width as a function of length in cambium cells and in parenchyma (see Table 1).

(*c*) *Cambium: Phloem Parenchyma Antagonism*

For a strip containing a layer of cambium against a layer of parenchyma changes in the overall shape of the strip will accompany changes in the turgor of the cells. The cambium will manifest most of any volume change by lateral expansion or contraction; but the behaviour of the parenchyma is not so readily predicted, the change in shape being a complex function of turgor. For if we calculate the change in tangential surface as a function of volume and increasing cell length, we obtain as the first differential of

$$S = \tfrac{1}{2}L(p+W)$$

$$\frac{S}{L} = \frac{(c+2p)(c-p)}{c}, \text{ where } c^2 = 4p^2 - L^2,$$

or

$$\frac{S}{L} = \frac{(W+p)(W+2p)}{(W-p)}.$$

The value of this differential varies from $2p$ to $-\infty$ as shown in Table 2.

This means that an originally water-saturated parenchyma cell (maximum surface $S = 3p^2$) may either shorten or lengthen, depending on the tissue tension.

Conversely a non-saturated cell may either lengthen or shorten when taking up water. It will lengthen if $L > p\sqrt{3}$ and shorten if $L < p\sqrt{3}$. As observation of this change of shape is difficult in the plant we do not know whether there is any normal preference; but as the parenchyma is apparently granted "two degrees of freedom" and the cambium only one, it seems reasonable to assume that in the strips, at least, it is the cambium that determines changes in orientation, and the phloem parenchyma (within limits) accommodates itself to these changes.

TABLE 1

W AND L VALUES CALCULATED FOR CAMBIUM AND PARENCHYMA

(*a*) Cambium

W	L	Assuming $a = 50\mu$ and $b = 150\mu$	
		W (μ)	L (μ)
0·0 a	1·000 $a+b$	0	200
0·1	0·995	5	199·7
0·2	0·978	10	198·9
0·3	0·952	15	197·6
0·4	0·916	20	195·8
0·5	0·866	25	193·3
0·6	0·800	30	190·0
0·7	0·715	35	185·8
0·8	0·600	40	180·0
0·9	0·138	45	169·0
1·0	0·000	50	150·0

(*b*) Parenchyma

W	L	Assuming $p = 11{\cdot}5\mu$	
		W (μ)	L (μ)
3·0 p	0·00 p	34·5	00·0
2.8	0·88	32·2	10·1
2.6	1·20	30·0	13·8
2.4	1·43	27·6	16·5
2·2	1·60	25·4	18·4
2·0	1·73	23·0	19·6
1·8	1·83	20·8	21·0
1·6	1·92	18·4	22·1
1·4	1·96	16·2	22·6
1·2	1·99	13·8	22·9
1·0	2·00	11·5	23·0

In homogeneous two-tissue strips, if they are long compared with the individual cells, curvature accompanying turgor changes will be constant throughout. As a result, strips placed in water curve towards a circular form — curvatures other than circular occur only if the strip is of uneven thickness or an antagonist has been damaged. In addition to longitudinal curvature due to a contraction in length of the cambial cells (Table 1), there will be a lateral curvature due to their expansion in width. Measurements directly made on the cambium cells can be used to assess the lateral curvature while a method for measuring the longitudinal curvature is given below. The appearance of strip, say 30 by 2 mm, turgid in water is like the circumference of a circle; the inner surface (cambium) is convex in cross section.

TABLE 2

L AND S/L VALUES CALCULATED FOR PHLOEM PARENCHYMA AS A FUNCTION OF p

L	S/L
0·0 p	2·00 p
0·2	1·99
0·4	1·94
0·6	1·86
0·8	1·75
1·0	1·57
1·2	1·35
1·4	1·03
1·6	0·53
1·73 ($\sqrt{3}$)	0·00
1·8	−0·043
1·9	−0·161
1·95	−0·300
2·00	$-\infty$

Only if the strip is homogeneous throughout will the radius of curvature be constant along the strip; then to measure this radius is a means of assessing the longitudinal curvature. Suppose the strip originally to have had a length L; now if it becomes a chord D of a circular circumference, it is evident that

$$D = 2r.\sin(\pi - L/2r),$$

where r is the radius of curvature.
For a circle, the curvature

$$C = 1/r,$$

so that

$$D = \frac{2\sin(\pi - \frac{1}{2}LC)}{C}.$$

(*d*) *Other Preparations*

Other preparations were considered in much the same way as cambium-phloem; however, they all proved rather rigid except the xylem-medulla system which produces a tight, helicoidal structure not unlike a tendril in certain climbing plants. The linear expansion of medullary cylinders was also measured. Details are presented with the results.

III. EXPERIMENTAL RESULTS

(*a*) *Cambium-phloem Strips*

The strips are sufficiently thin to arrive at an equilibrium in water within 15 or 20 min, although after 24 hr there may be some readjustment. There was usually no significant trend in tensions between internodes, but variability in the youngest internodes is higher, presumably because of immaturity in the cambium. From a plant with 11 internodes 10-12 strips could be prepared from internodes numbered 2-9 inclusive. The length of each strip was 30 mm. Results in this case are given as the length of the chord *D* (Table 3).

TABLE 3

DISTRIBUTION OF TENSION ALONG THE STEM

D (mm)	Internode Number, from Top								Totals
	2	3	4	5	6	7	8	9	
9-10	1								1
11-12	1								1
13-14		1		1		4	1		7
15-16	1		1			3		3	8
17-18	2	1		1		1	1	4	10
19-20	2	3	1	4	3	1	1	1	16
21-22	3	3	4	2	5	1	3	2	23
23-24		1	1	4	4	1	4	2	17
25-26	1		4						5
27-28	1	2	1						4
N	12	11	12	12	12	11	10	12	92
$\bar{D}$	19·0	20·8	22·8	20·5	21·7	16·6	20·9	18·7	20·15
$3\sigma_D$	4·38	3·41	2·82	2·0	1·44	3·36	2·94	2·64	2·62

That the nature of the release is dependent on the physiological condition of the material is illustrated in Table 4, where the reaction of an "acclimatized" plant is compared with a control. The effect of the osmotic pressure of the solution is also illustrated. Here curvature is expressed as the reciprocal of the radius.

As the turgor of the cambium controls curvature, the pretreatment *B* must increase osmotic pressure or alter the plasticity of the cambium cell walls, so increasing suction pressure and producing greater volume at turgidity than in control tissue.

TABLE 4

VALUES OF $1/r$

Treatment of Strips	Paraffin Oil	Water	Glucose 0·1M	Glucose 0·3M	Glucose 0·5M
Plant *A* outside at about 16°C	0·08	0·39	0·32	0·12	0·00
Plant *B* 48 hr incubated at 27°C	0·13	0·47	0·38	0·12	0·07

It is clear from Table 4 that the osmotic gradient affects the value of $1/r$. The osmotic systems of cells only affect the shape of tissues when turgor pressure is actually present. As we are concerned with volumes rather than pressures it is desirable to use the following terminology to cover changes in volume between the point of incipient plasmolysis and full tissue turgor. Any isolated tissue can be considered as saturated to a degree corresponding to its turgor; the volume of cells as isolated minus the volume when plasmolysed is the *saturation factor* (S_f). The volume at full saturation (turgid) minus the volume as isolated gives the *saturation deficit* (S_d). And the difference in volume between the fully saturated and plasmolysed states may be considered as the *total saturation capacity* (S_c) of the tissue, and represents the full change in volume that can be brought about by the turgor pressure of the protoplasts. Experimentally, the tissues may be measured in paraffin oil, to give V_o, in water (V_w), and in a plasmolyte such as glycerol to give V_p. We can then determine the saturation states as follows:

$$S_f = V_o - V_p \ , \ S_d = V_w - V_o \ , \ S_c = V_w - V_p.$$

As the release of tensions amounts in fact to an adjustment to new volume relationships, these being partly determined by the osmotic gradient, we may consider a strip about to become turgid in water to be capable of a certain tension release corresponding to the factor

$$S_f/S_c\,.$$

This may be expressed as a percentage release as in Table 5. In the experiments volumes were not obtained, but as already pointed out they have been assumed to be proportional to linear dimensions. Our two methods of measurement, by the calculation of $1/r$ for longitudinal curvature, and by calculating mean cell width by direct counting of cambium cells on a calibrated field for transverse curvature, correlate well in calculating tension releases.

Table 5 gives the results of measurements by both methods in different solutions, including some done in 10 p.p.m. 2,4-D. Apart from the lack of systematic distribution of the tensions, previously seen in Table 3, the effect of 2,4-D in releasing tensions is of interest. Before considering further results obtained with 2,4-D on cambium-phloem strips, some observations on the behaviour of other tissue preparations are presented.

(*b*) *Medulla Cylinder*

The living pith in the plant is under compression (Fig. 6) as seen by the wavy outline of the tannin cells. In very old internodes the pith cells are sometimes dead, and the tannin ducts show brown contents. Radial sections exposed to the air gradually turn brown where these ducts are present. If a pith cylinder is removed with a narrow cork-borer (we used 2.5 mm diameter) it will elongate as a rule over 10 per cent. of its original length in situ. Care must be taken not to take any adjacent xylem with the cylinder or the preparation becomes very contorted. Swelling in water continues for several hours.

TABLE 5

Internode (from top)		Curvature of Strip		Average Width of Cambium Cells (μ)				Percentage Release of Tension	
No.	*L* (mm)	Water 1/*r*	2,4-D 1/*r*	Water	2,4-D 10 p.p.m.	Glycerol 50%	Paraffin Oil	Water (compared widths)	2,4-D 10 p.p.m. (compared widths)
3	27			11·8	10·0	9·0	9·2	7	33
4	26	0·52	0·46	13·2	9·6	7·6	9·2	28	36
5	20	0·58	0·54	18·0	13·8	13·0	12·7	6	51
6	16	0·48	0·52	19·6	14·5	11·6	13·0	18	36
7	17	0·40	0·40	19·5	19·4	14·5	17·2	51	98
8	24	0·41	0·40	18·0	15·5	11·4	12·7	20	64
9	31	0·57	0·55	20·6	18·0	15·0	16·5	28	38
10	29	0·55	0·45	25·4	23·6	19·5	21·2	29	70
11	32	0·44	0·44	24·0	21·2	16·8			61
12	35	0·24	0·24	24·0	21·0	13·8	20·6	68	70
13	45	0·68	0·59	21·5	18·0	16·0	19·0	55	36
14	50			28·2	24·0	14·2	20·6	46	70
15	47	0·62	0·57	25·4	14·0	13·4	14·4	8	8
16	66	0·58	0·62	21·0		12·7	13·8	13	
Average		0·52	0·48					29	49

TABLE 6

PERCENTAGE INCREASE IN LENGTH OF MEDULLARY CYLINDERS

(Temperature 24·5°C)

Time (min)	0	20	55	85	125	215
Water	0	13	24	30	34	40
10 p.p.m. 2,4-D	0	10	15	15	15	13

Considering its striations the medullary parenchyma is highly disposed to increase in length rather than breadth. Results obtained are given in Table 6 and are also shown in Figure 9.

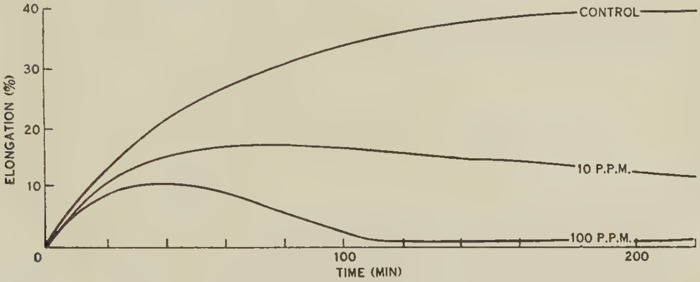

Fig. 9.—Influence of various concentrations of 2,4-D on the elongation of medullary cylinders as a function of time at 24.5°C.

Measurements were made both by direct observation, measuring, and by recording on a kymograph; however, no extensive work was done on these preparations.

(*c*) *Medulla-xylem Strips*

These show a much greater tendency to curl up on saturation than do the cambium-phloem strips; this is because of the initially high compression of the pith and the inextensible nature of the xylem tissue. Satisfactory preparation is possible only before secondary thickening. The strips suffer disadvantages of heterogeneity and slow adjustment. Results are given in Table 7.

Table 7

LENGTH (MM) OF CHORD *D* OF MEDULLA-XYLEM STRIP AFTER VARIOUS TIMES

Five strips/batch

Time (min.)	0	20	50	130	300
Water	25	3·4	−4·0	−8·2	−10·8
100 p.p.m. 2,4-D	25	4·0	−1·3	−1·5	−1·8

(*d*) *Cortical Preparations*

By taking transverse sections of the tissues sheathing the xylem cylinder, small slit circlets are obtained, and their reactions can be examined. These rings open out a little in water and less in 2,4-D and glucose solutions. In one experiment the mean opening between the ends of five rings in water was twice that of a replicate set in 10 p.p.m. 2,4-D, after 20 min. Although equilibrium had not occurred, there was a similar difference after 1 hr. Complexity and heterogeneity are again disadvantages.

Longitudinal strips of pericycle and adhering cortex show only slight curvatures in water and, as errors in the estimation of $1/r$ in the range 0-0.1 are large, these preparations were not studied further. The great rigidity of the pericyclic fibrous tissue is responsible.

TABLE 8

LENGTH (MM) OF CHORD *D* FOR THREE TISSUE-TENSION SYSTEMS IN WATER AT 17°C

Time (min.)	0	5	15	30	60	90
Medulla-xylem	25	20·6	11·8	6·1	1·8	0·1
Cambium-phloem	25	10·3	8·5	8·1	8·3	8·2
Pericycle-cortex	25	23·0	22·3	22·8	22·5	22·8

Table 8 compares the curvatures of three tissue-tension systems in water, five strips per figure, temperature 17°C. These values are the basis of the curves in Figure 10.

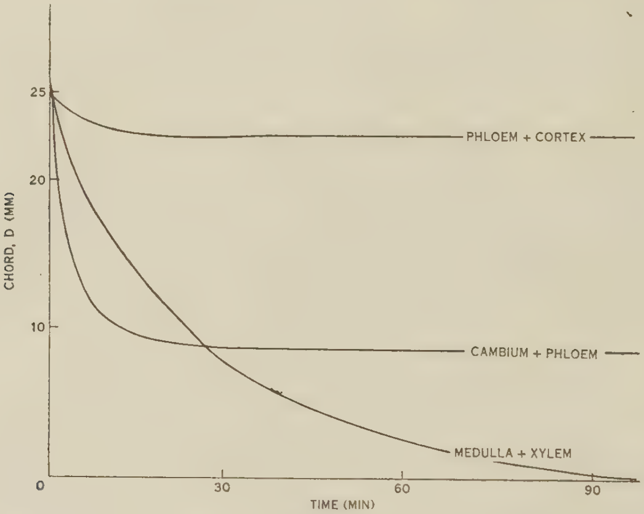

Fig. 10.—Comparison of curvature (measured as chord) as a function of time in various preparations from the same internode at 17°C.

(*e*) *Effects of* 2,4-*D and IAA on Cambium-phloem Strips*

The appreciable effect of 2,4-D in lowering the percentage release of tension in cambium-phloem strips (Table 5) was investigated with respect to concentration of 2,4-D and temperature. The effect on the value of 1/*r* is presented in Table 9. It is seen that while the effect of 2,4-D is reduced at lower temperatures, even here the curvature is determined by the amount of 2,4-D present (Fig. 11).

In Figures 12 and 13 the differences in the values of 1/*r* for strips in 2,4-D or IAA and water are plotted. Notable features are the rapidity with which differences become apparent and the tendency for the IAA set gradually to approach the water value; there is no such tendency to reversal in 2,4-D.

Strips plasmolysed in sucrose plus 2,4-D attain equilibrium much faster than those in sucrose alone. This effect is shown in Figure 14. The width of the cambium cell was established by counting the number of cells over a known distance on an irrigation slide. The concentration of 2,4-D was 0.5 p.p.m.*

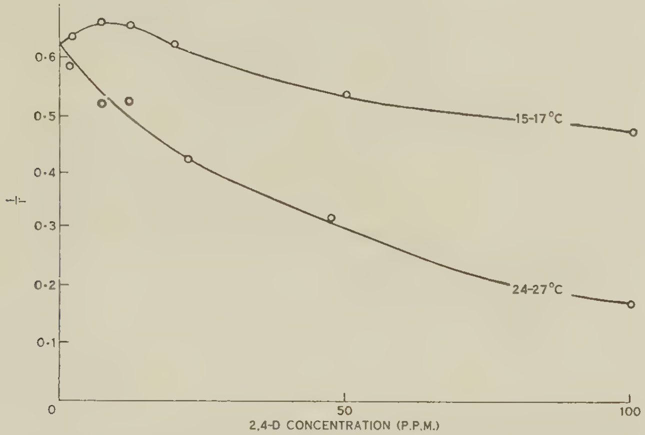

Fig. 11.—The effect of temperature on the release of tensions at various concentrations of 2,4-D.

In no case has tissue already turgid in water shown measurable alteration in volume on being transferred to solutions of 2,4-D; although work in progress indicates that this can occur. No such effect could be found for cambium in 10^{-3} to 10^{-9}M 2,4-D. Quite frequently, however, disappearance of air from intercellular spaces was observed.

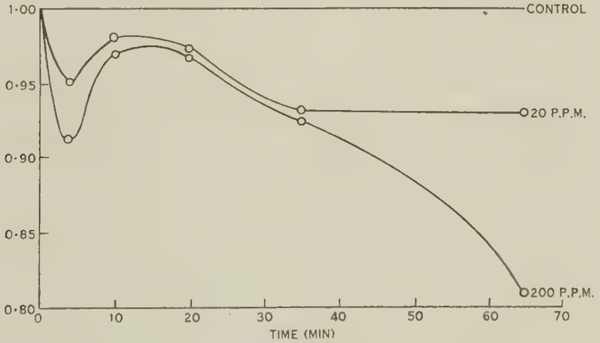

Fig. 12.—Curvature of strips as a function of time at 20.8°C for 20 and 200 p.p.m. 2,4-D expressed in fractions of the curvature of the control.

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* As a bioassay, the cambium-parenchyma strip is much less sensitive than an assay by means of root growth (5×10^{-7} p.p.m. as against 10^{-12} p.p.m for the cress root) (Moewus 1948, 1951).

IV. Discussion

The fact that cell elongation in growth is dependent upon a temporary excess of turgor pressure over wall pressure was recently emphasized by Burstrøm (1948, 1951) and Haines (1951). While the systems described in this paper do not contain actually growing cells, they resemble growing tissues in their natural tendency to increase in volume and alter in shape, owing to the release or modification of the wall pressure through tissue tensions. The

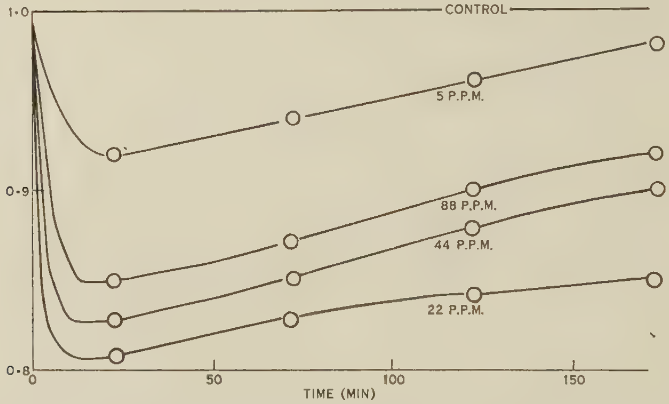

Fig. 13.—Curvature of strips as a function of time at 25°C for various concentrations of IAA expressed in fractions of the curvature of the control.

Table 9

CURVATURES AS $1/r$, BROUGHT TO STANDARD FOR WATER (COMPOSITE OF MANY EXPERIMENTS)

2,4-D (p.p.m.)	Temperature (°C)						
	27	26	25·5	24	17	16	15
0·0	0·62	0·62	0·62	0·62	0·62	0·62	0·62
1·0	0·56		0·61	0·57			
1·5			0·61				
3·0		0·53			0·53		
4·0				0·56			
5·0		0·38				0·62	0·64
6·0		0·53		0·51			
10·0			0·56		0·60	0·66	0·66
12·5		0·54					
15·0				0·50			
20·0					0·52	0·60	0·67
25·0		0·49	0·34				
50·0			0·49				
100·0	0·12	0·15	0·28		0·49		

changes in volume are determined by the turgor pressure and the previous shape of the cells. Schneider (1942) recognized in the pea test the importance of cell shape to curvature; yet he ascribed the curvature to obscure "nastic" and "traumatic" responses which he considered affected growth. This seems to cloud the issue, however, for the nastic and traumatic responses seem to be inseparable theoretically from the change in tissue tensions accompanying the preparation of the epicotyl and its immersion in water, and the "growth" may be a local increase in turgidity accompanying water saturation.

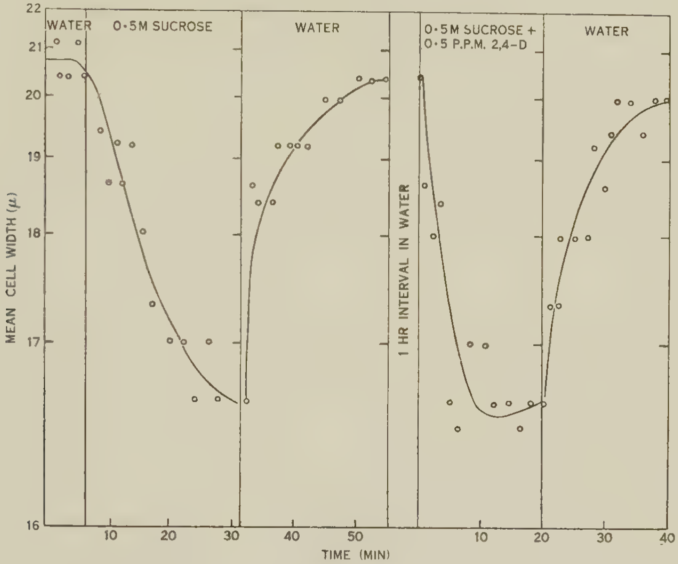

Fig. 14.—Plasmolysis and deplasmolysis as a function of time of cambium cells with and without 0.5 p.p.m. 2,4-D at 19-21°C. Logarithms of cell width are plotted to enhance the difference between plasmolysis and deplasmolysis.

In view of the effect of auxins on the release of tensions in *Bryophyllum* strips, we believe that their physiological action is directly on turgor adjustment, and consequently that their effects in plants will be felt wherever active turgor adjustment is going on. This also occurs throughout the plant to a limited extent, but is most obvious in growing regions and in photosynthetic cells and wounded tissue. Even in tissues apparently in equilibrium with their environment, such as thin potato discs washed in water for 24 hr, there is a gradual increase in turgor in auxin solution (Reinders 1938, 1942; Hackett 1951), and in unpublished experiments with beetroot discs there was sometimes a decrease in turgor. The "paralysing" effect of 2,4-D on pulvinar movements in *Oxalis* (Baas Becking and Everson 1952) and the familiar hypo- and epinastic movement in leaves can be considered in the light of turgor adjustments; while an "internal wilt" like that described by Gaümann (1949) and co-workers for parasitized plants, a flaccidity without loss in weight, has also been noticed by us in 2,4-D-treated leaves of *Bryophyllum*.

In Table 5 the greater percentage release of tensions in 10 p.p.m. 2,4-D is observed; in 2,4-D the tissues change their volume less in coming to equilibrium. This effect is comparable with that of placing strips in dilute sugar solutions (Table 4) where the smaller osmotic gradient produces less ultimate distension than in water for cells to attain equilibrium. And in experiments where tissue was plasmolysed in the presence of 2,4-D, the rapid plasmolysis suggests a higher effective osmotic pressure gradient than in the control. However, the effect could be due to any of the three following processes:

(*a*) Increased permeability of the protoplast,

(*b*) Increased wall pressure,

(*c*) Increased osmotic value of the vacuolar sap,

of which (*a*) seems the most feasible considering the rate of the adjustments concerned. von Guttenberg and Beythien (1951) and Pohl (1948) have demonstrated effects of auxins on the permeability of *Rhoeo* epidermis and the *Avena* coleoptile respectively; and Veldstra (1947) has suggested a possible mode of action in this respect by his study of the surface characteristics of auxin molecules.

The change in width of cambium cells in cambium-phloem strips is thought to be directly caused by turgor changes in the cambium rather than in the phloem, as discussed earlier; and while further experiment would be helpful, it is now suggested that an abnormal release either of water or of cell sap occurs when auxin acts on a protoplast already changing in volume. In the experiments with strips, subturgid cells were either becoming turgid or plasmolysed. If water is extruded from the protoplast, as is claimed to occur under pressures greater than 10 atmospheres (Bennett-Clark and Bexon 1940),* this could be brought about by a change in the surface area of the tonoplast with a concomitant increase in the osmotic pressure of its contents. If water were extruded this would require a great deal of energy. In this case the re-establishment of the equilibrium, however, would be exergonic. The opposite is the case when cell sap is extruded, where the re-establishment of the equilibrium, by intake of salt, would require energy. In the latter case, however, intracellular adjustment might play a role (e.g. starch-sugar equilibrium). The entry of vacuolar substance into the cytoplasm, moreover, might extend a wide influence on metabolic paths, due, in part, to an increase in available substrate with little change in osmotic pressure.

For these reasons we assume that there is more probability that cell sap is extruded rather than water. This cell sap may leave the cytoplasm and appear in the intercellularies. In this connection the experiments of Nicolai (1929), who obtained tumorous growths on roots mechanically stimulated and noticed a coincidental extrusion of air from the intercellular spaces, are recalled.†

The changes in shape of cells in the tissue tension systems studied may be contrasted with the effects of hygroscopic tensions observed by Diehl *et al.*

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* This seems unlikely on thermodynamic grounds, however.

† On these and similar, as yet little-exploited phenomena, see Prillieux (1868), Hofmeister (1860), Janse (1926).

(1939). In systems dependent on tensions of osmotic origin the properties of the tonoplast are most important.

No effect of 2,4-D on the plasticity of the cell wall could be found with sagging, plasmolysed cambium-phloem strips or with cylinders cut from the medulla (after Heyn 1934). The short-term experiments described in this paper may be considered independent of possible subsequent alteration of the structure of the cell wall, which are inherent in growth processes.

V. Acknowledgment

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THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

V. SOLUBLE NITROGEN CONSTITUENTS

By H. S. McKee* and Gerda E. Urbach*

[*Manuscript received February* 9, 1953]

Summary

The ninhydrin-reacting compounds of the apple fruit have been studied during its development on the tree. The compounds present show little variation, but glutamine, conspicuous in very young and in over-mature apples, disappears at intermediate stages. The increase in soluble nitrogenous compounds occurring in over-mature apples left on the tree (Pearson and Robertson 1953) is confirmed qualitatively. An account is given of the chromatographic behaviour of several unidentified ninhydrin-reacting substances found in extracts of apple fruits, leaves, and stems.

I. Introduction

Previous communications from this laboratory (Robertson and Turner 1951; Pearson and Robertson 1953) have reported that in the developing Granny Smith apple cell division is completed about 6 weeks after blossoming, subsequent growth being due to cell enlargement. Total nitrogen and protein nitrogen per cell are correlated with cell surface rather than cell volume, suggesting that the enlarging cell maintains a layer of protoplasm of approximately constant thickness.

Up to the time at which the fruit is normally picked there is a high correlation between the amounts of protein nitrogen and of soluble nitrogen per cell, but in fruits left longer on the tree soluble nitrogen increases faster than protein nitrogen (Pearson and Robertson 1953). The present paper reports a qualitative study of the ninhydrin-reacting substances of the Granny Smith apple during its development on the tree. These substances were extracted with alcohol, adsorbed on "Zeo-Karb" 215, eluted with ammonia, and identified by paper chromatography; the technique used detected only large changes in concentration. Similar studies were made with leaves and branches.

II. Material and Methods

The work was carried out in the 1950-51 and 1951-52 seasons, results in both being similar. The data cited in the paper, unless otherwise stated, refer to the later season.

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(*a*) *Sampling*

All the fruits, and some of the leaves and branches analysed in this work, came from one tree at Bathurst, N.S.W. This tree also supplied part of the fruit used in earlier studies. Sampling dates and other details are shown in Table 1.

Table 1

SAMPLING DATES

Pick No.	Date 1950-51	Days from Full Blossom	Date 1951-52	Days from Full Blossom
1	26.xi.50	54	3.x.51	0
2	18.xii.50	76	10.xi.51	38
3	15.i.51	104	7.i.52	96
4	12.ii.51	132	18.ii.52	138
5	12.iii.51	160	25.iii.52	174
6	9.iv.51	188	5.v.52	215
7	7.v.51	216	23.vi.52	264
8	4.vi.51	244	7.vii.52	278
	18.vi.51 Tree bare, fruits and leaves picked up from the ground	258		

The sampling technique was designed (Pearson and Robertson 1953) to provide a statistically random sample of fruits from all parts of the tree. Leaves for analysis were picked immediately behind fruits on the experimental tree or on other Granny Smith trees. At 244 days from full blossom (1950-51), when most of the leaves had already fallen, the sample of leaves was taken from all parts of the tree. When the last sample was taken in 1950-51 the tree was already bare of fruits and leaves, and those analysed were picked up from the ground beneath it, the leaves being already brown and moist.

(*b*) *Extraction*

The material was dropped into absolute ethanol as soon as possible after cutting. At 0 days the complete flowers were used, and at 34 days the whole fruit less seeds, but later the cortex was analysed separately. About 100-400 g of tissue was taken for analysis of mature fruits and ethanol added to a final concentration of about 75 per cent. The tissue was then ground in the alcohol for 5 min in a Waring Blendor and the homogenate filtered through a Whatman No. 31 filter-paper on a Buchner funnel without pressure.

(*c*) *Preparation of the Extract for Chromatography*

The ethanolic extract was passed down a column of "Zeo-Karb" 215 (10 g). The presence of ethanol did not interfere with adsorption of the amino acids. The column, prepared by the method of Partridge and Westall (1949), was first treated with aqueous alcohol of the same concentration to prevent precipitation of chlorophyll. It was eluted with ammonium hydroxide (approximately

1N) until the eluate no longer gave a reaction with ninhydrin. The column was then eluted with 2.5N sodium hydroxide and the eluate tested for arginine (Sakaguchi 1925). The ammonia eluate was evaporated to dryness under reduced pressure, taken up in 2 ml of water per 100 g wet material, and filtered. The filtrate was then ready for chromatography.

(*d*) *Protein Hydrolysis*

The residue filtered from the ethanolic extract was washed several times with 75 per cent. aqueous ethanol and hydrolysed under reflux for 24 hr with 5N hydrochloric acid. The hydrolysate was filtered, evaporated to dryness, taken up in about 20 ml of distilled water, refiltered, and passed through a "Zeo-Karb" 215 column to remove excess hydrochloric acid and other non-basic impurities. Subsequent procedure was similar to that described above for tissue extracts.

(*e*) *Chromatographic Technique*

(i) *Solvents.*—The ascending method of Williams and Kirby (1948) was used as modified by Wolfson, Cohn, and Devaney (1949). For two-dimensional chromatograms 80 per cent. phenol was followed by a mixture (4 : 1 : 1 by volume) of butyl alcohol, acetic acid, and water (Reed 1950); in early work the non-aqueous phase of the corresponding 4 : 1 : 5 mixture (Partridge 1948) was used. Aqueous ethanol (75 per cent.) (Patton and Foreman 1949; Bentley and Whitehead 1950) was occasionally used as second solvent. Phenol was removed by drying at 40-50°C. The butyl alcohol-acetic acid was removed by heating similarly, or by air-drying for several hours. The papers were then sprayed with ninhydrin (0.1 per cent. in absolute ethanol) and placed at 40°C, the temperature being raised to about 100°C in 45 min. The chromatogram was examined frequently, each spot being outlined when it first appeared in order to separate spots which overlapped on further heating.

(ii) *Filter Paper.*—Whatman No. 1 paper was used whenever available. Ekwip No. 1, stated by the manufacturers (Industrial Equipment (A/asia.) Pty. Ltd., Paddington, N.S.W.) to be identical with Whatman No. 1, gave only about two-thirds of the rates of solvent ascent with Whatman No. 1, being equivalent to Whatman No. 2. The map of spots, however, was similar on all three papers.

(*f*) *Estimation of Amide Nitrogen*

Amide nitrogen was estimated by the method of Vickery *et al.* (1935) on aqueous extracts of dried tissue.

III. RESULTS

(*a*) *Identification of Individual Amino Acids*

The R_F values found with butyl alcohol-acetic acid are shown in Table 2. These values agree well with the unpublished data, for the same solvent, of Dr. T. M. Reynolds, of this Division.

Individual amino acids were identified using authentic specimens. A substance was considered to be identified only when it gave a single spot with an authentic specimen on two two-dimensional chromatograms prepared with phenol followed respectively by butyl alcohol-acetic acid and by 75 per cent. aqueous ethanol.

TABLE 2

R_F VALUES OF VARIOUS AMINO ACIDS IN THE NON-AQUEOUS PHASE OF BUTYL ALCOHOL-ACETIC ACID-WATER (4 : 1 : 5)

Amino Acid	R_F
Asparagine	0·09
Arginine	0·14
Aspartic acid	0·20
Serine	0·23
Glycine	0·24
Threonine	0·25
Glutamic acid	0·25
β-Alanine	0·27
α-Amino-*n*-butyric acid ..	0·37
α-Amino-*iso*butyric acid ..	0·37
Valine	0·47
Phenylalanine	0·57
Leucine	0·62

The colour reactions of isatin (Grassman and von Arnim 1935; Acher, Fromageot, and Jutisz 1950; Hulme and Arthington 1952) with amino acids were also studied, results being shown in Table 3.

(*b*) *Unknown Ninhydrin-reacting Substances*

In the various extracts examined, three unidentified ninhydrin-reacting substances occurred regularly, and several other spots appearing occasionally are believed to indicate further unidentified constituents. Those occurring regularly will be cited as unknowns *A*, *B*, and *C*. They form distinct spots when chromatographed with added anserine, carnosine, citrulline, ornithine, arginine, lysine, histidine, N-methylhistidine, sarcosine, theanine (ethylglutamine), γ-methyleneglutamine, baikiain (1,2,3,6-tetrahydropyridine-2-carboxylic acid), nipecotinic acid, and putrescine.

In phenol *A* runs just ahead of valine, and in butyl alcohol-acetic acid level with threonine; it gives a yellow colour with ninhydrin and a blue colour with isatin. *A* also gives a blue spot with a spray containing nitroprusside and acetaldehyde (Feigl 1939). These colour reactions are all consistent with its being an imino acid. In phenol *B* runs level with *A* and just ahead of it in butyl alcohol-acetic acid; it gives a purple colour with ninhydrin. In phenol *C* runs just behind *B* and in butyl alcohol-acetic acid level with asparagine; it gives a yellow-brown colour with ninhydrin.* A purple spot overlapping *C* on standard ninhydrin-sprayed two-dimensional chromatograms, but displaced in phenol-ammonia to a position ahead of proline, is attributed to arginine. This identification was made after most of the work reported in this paper had been completed. It is based on the overlapping of the spot in question with that of added arginine in three separate solvents, and on the deep red spot formed in the same positions on chromatograms sprayed with a modified Sakaguchi reagent (Roche *et al.* 1951). Arginine is incompletely eluted from "Zeo-Karb" 215 by ammonium hydroxide. Its distribution in the samples examined is therefore uncertain, but the greatest concentrations are found in the bark.

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* Note added April 1953: recent tests with diazotized *p*-bromaniline indicate that **C** is histidine.

TABLE 3

COLOUR REACTION OF VARIOUS AMINO ACIDS WITH ISATIN

Amino Acids	Colour
Aspartic acid . . . . Glutamic acid	Dark purplish blue
Threonine . . . . Serine . . . . . . Tyrosine	Light brown
β-Alanine . . . . γ-Aminobutyric acid . .	Dark blue
Proline . . . . Hydroxyproline . .	Bright blue
Pipecolinic acid . .	Bluish green
Asparagine, glutamine, valine, leucine, glycine, alanine	Pink

(*c*) *Individual Amino Acids in Fruit*

The free amino acids detected in the apple fruit at various times during its development are shown in Table 4. The most prominent in every sample was asparagine, although it crystallized from concentrated extracts before chromatography. The other prominent amino acids were aspartic acid, glutamic acid, serine, threonine, and alanine. Valine, proline, γ-aminobutyric acid, methionine sulphoxide, and "leucine" were also present in all samples; "leucine" is one or both of isoleucine and leucine, which were not separated by the technique used. Nearly all the amino acids showed a high concentration at 0 days (blossom), dropping sharply at 38 days, with a slight further drop at 96 days, after which little change appeared until 264 days, when there was a considerable increase, though not reaching the initial level. Thus over the period of increasing cell size (Robertson and Turner 1951) their concentration remains approximately constant. The late increase in concentration occurs after cell enlargement ceases.

Minor components were demonstrated in chromatograms of more concentrated extracts of fruit from some picks, and are recorded in Table 4. As asparagine crystallized out before chromatography, changes in its concentration cannot be deduced from the chromatograms.

Table 4

AMINO ACIDS DETECTED AT VARIOUS STAGES OF THE DEVELOPMENT OF THE GRANNY SMITH APPLE FRUIT (1951-52 SEASON)

Days after Full Blossom	Aspartic Acid	Glutamic Acid	Asparagine	Glutamine	Serine	Alanine	Tyrosine	Valine	Leucine and/or Isoleucine	Proline	β-Alanine	Threonine	γ-Aminobutyric Acid	Hydroxyproline	Pipecolinic Acid	Methionine Sulphoxide	Phenylalanine	Glycine	Unknowns *A*	Unknowns *B*	Unknowns *C*
Sample Fruit																					
0	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
38	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	×
96	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
138	×	×	×	—	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
174	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
215	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	×
264	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
278	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	—	—	×	×	×
Leaves																					
0	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	—	—	×	×	—
38	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	—
96	×	×	×	×	×	×	×	×	×	×	—	×	×	—	—	×	—	—	×	×	—
138	×	×	×	×	×	×	—	×	×	×	×	×	×	—	×	×	×	—	×	×	—
174	×	×	×	×	×	×	×	×	×	×	×	×	×	—	—	×	×	—	×	×	—
215	×	×	×	×	×	×	—	×	×	×	—	×	×	—	—	—	×	—	×	×	—
Branches																					
215	×	×	×	×	×	×	×	×	×	×	×	×	×	—	×	×	×	—	×	×	×
Hydrolysate of protein from fruit 264 days	×	×	—	—	×	×	×	×	×	×	—	×	—	×	—	×	×	×	—	—	—

× = Present; — = not detected by technique used.

The trend described above holds for aspartic acid, alanine, serine, threonine, and γ-aminobutyric acid, and particularly for "leucine" and valine. Glutamine shows the trend in an exaggerated form, being prominent in early and late picks, and faint at other times; it could not be demonstrated, even in concentrated extracts, at 138 days. Pipecolinic acid was present in all samples but was prominent only at 0 and 38 days. Methionine sulphoxide was always present; methionine sulphone sometimes occurred in small amounts. Both these may be artifacts produced by oxidation of methionine. Methionine sulphoxide increased steadily relative to the other amino acids in the course of each season, but was never a major constituent. Phenylalanine reached its greatest prominence at 96 days, and decreased later. β-Alanine was present always as a minor constituent, as were unknowns *B* and *C*. The yellow spots of proline and unknown *A* made it hard to compare their concentrations with those of the other amino acids; both were always present.

(*d*) *Amino Acids in Leaves*

Leaves differed from the fruit mainly in having less asparagine and more glutamine. On a wet-weight basis the amino acids were always less concentrated in leaves than in fruit. Asparagine crystallized from extracts of fruit but not of leaves. All the amino acids found in the fruits were found in the leaves also, though unknown *C* was found only in the earlier season. The most prominent were aspartic acid, asparagine, glutamic acid, glutamine, serine, alanine, threonine, valine, "leucine," γ-aminobutyric acid, and unknown *A*, which was more prominent compared with proline in the leaves than in the fruit. The concentration of all amino acids was less at 216 days in the 1950-51 season than in earlier picks and less still at 244 days, when aspartic acid was missing. In fallen leaves picked up at 257 days beneath the bare trees no amino acids could be identified, though the extract gave a faint colour with ninhydrin. A similar decrease had started by 215 days in the 1951-52 season; alanine gave a prominent spot, glutamic acid and asparagine small spots; but no other amino acids showed more than a trace. The decrease in amino acids in the leaves may, as suggested by Pearson and Robertson (1953), be related to the increase in amino acids in the fruit.

(*e*) *Amino Acids in Branches*

All the amino acids recorded for the fruit were found also in tthe branches, asparagine, alanine, glutamine, and sometimes unknowns *A* and *C* being particularly prominent.

(*f*) *Amino Acids in Protein Hydrolysates*

Acid hydrolysates of fruit protein at 188 and 216 days (1950-51) and 257 days (1951-52) showed many of the amino acids found free in the fruits, leaves, and stems. The most notable differences were the presence in the protein of glycine and hydroxyproline, and the absence of pipecolinic acid, β-alanine, γ-aminobutyric acid, and all the unknowns. Tyrosine was more prominent in the protein than in the free amino acids. Asparagine, glutamine, and tryptophane, if present in the protein, would be destroyed by acid hydrolysis.

(*g*) *Changes in Amide Nitrogen*

Amide nitrogen was estimated on dried samples of the last four picks of the 1950-51 season. The results are shown in Table 5. The last five columns in this table are derived from the data of Pearson and Robertson (1953).

IV. Discussion

The amino acids of apple fruits have been studied also by Joslyn and Stepka (1949), Hulme and Arthington (1950*a*, 1950*b*, 1952), and Hulme (1951). Joslyn and Stepka (1949) used dilute extracts from which sugars were not removed; they recorded only asparagine, aspartic acid, serine, γ-aminobutyric acid, valine, and leucine, but the failure to detect other amino acids cannot establish their absence. The amino acids found by Hulme and Arthington (1950*a*) in Bramley's Seedling apples included glycine and tryptophane, neither found as yet in Granny Smith. The amino acids found in Granny Smith but not in Bramley's Seedling are threonine, tyrosine, and the unknowns mentioned earlier. Hulme and Arthington (1950*b*) report glycine also in the varieties Worcester Pearmain and Cox's Orange Pippin, which both contain hydroxyproline, not yet found in Granny Smith. Hulme (1951) reports in the acid hydrolysate of the protein of Bramley's Seedling fruits all the amino acids, except hydroxyproline, recorded here for a similar preparation from Granny Smith; he found also arginine and lysine. Pipecolinic acid, already known from the apple (Hulme and Arthington 1952), is a recently recognized plant constituent which may be generally distributed. It has been recorded in several species by Morrison (1952) and by Zacharius, Thompson, and Steward (1952) and has since been found (Morrison, personal communication) in 25 out of 33 angiosperms examined.

Table 5

AMIDE NITROGEN IN RELATION TO OTHER NITROGEN FRACTIONS (1950-51 SEASON)

Days After Full Blossom	Amides as % Dry Weight	Amides as % Total Nitrogen	Amides as % Soluble Nitrogen	Amide Nitrogen ($g \times 10^{-10}$/cell)	Soluble Nitrogen ($g \times 10^{-10}$/cell)	Soluble Nitrogen Other Than Amide ($g \times 10^{-10}$/cell)
160	0·11	35	80	8	10	2
188	0·10	40	80	8	10	2
216	0·10	35	67	10	15	5
244	0·13	33	58	14	24	11

The present work has failed to detect glycine or the sulphur-containing amino acids, methionine, cystine, and cysteine, in alcohol extracts of Granny Smith apples. Of their oxidation products methionine sulphoxide occurs regularly and methionine sulphone sporadically. Methionine sulphoxide is found both free and in protein hydrolysates. It may be formed by the oxidation of methionine during analysis.

Pearson and Robertson (1953) found large increases in soluble nitrogen per cell at 216 and 244 days in the 1950-51 season, both samples being taken after the normal picking date for commercial fruit. Table 5, based on their results, shows that, though part of this increase is due to amides, there is also a fivefold increase in non-amide soluble nitrogen per cell. The material analysed may have lost some amide in drying, but since extracts of dried material contain glutamine, losses of the more stable asparagine are probably small. Since asparagine is the main amide present, losses of amide in drying are unlikely to be important. Moreover, at 160 and 188 days (Table 5) most of the soluble nitrogen is amide nitrogen. This is incompatible with extensive amide breakdown, as half of the amide nitrogen lost would appear in an amino acid, and much of the other half would probably be retained as ammonium salts of organic acids.

The compounds involved in the increase of soluble nitrogen in the later samples are not specified by the present work, which does, however, confirm qualitatively the earlier quantitative observations of Pearson and Robertson (1953). There is a general increase in both amides and non-amide amino acids but nitrogenous compounds not reacting with ninhydrin may also be involved.

Glutamine disappears about the same time as starch accumulation (Pearson and Robertson 1953) reaches its maximum, suggesting a possible connection between the metabolism of protein and of carbohydrate; its clarification, however, must await further work. The disappearance of glutamine is not due to a shortage of the glutamic acid used in its synthesis. If views expressed earlier (Robertson and Turner 1951) on the relations between phosphorylations and the synthesis of starch and other substances in the apple are correct, the reduced glutamine content may reflect a shortage of adenosinetriphosphate, known (Elliott 1948; Speck 1949) to be required for its synthesis *in vitro*.

The almost complete constancy of the amino acid pattern during the development of the apple fruit is a striking and rather surprising feature of the results. It appears that, even when intense protein synthesis occurs, the amino acids (except glutamine) likely to occur as residues in protein molecules are always in excess.

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THE GROWTH-PROMOTING EFFECT OF INDOLE-3-ACETIC ACID ON THE COMMON CULTIVATED MUSHROOM, *PSALLIOTA HORTENSIS* (COOKE) LANGE FORMA *ALBIDA* LANGE

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Summary

A strain of the white-capped form of the common cultivated mushroom, *Psalliota hortensis* (Cooke) Lange forma *albida* Lange, appeared unable to grow appreciably on a liquid basal medium which contained glucose, asparagine, and mineral salts. Addition of thiamin and biotin increased the growth a little. A number of other vitamins were without effect. Indole-3-acetic acid (IAA) in concentrations of from 5 to 100 mg/l markedly increased growth, particularly in the presence of thiamin and biotin, but only when the inoculum was floating on the surface of the culture medium. If the inoculum was submerged, addition of IAA had very little effect. The growth of both floating and submerged inocula was markedly increased by the addition of yeast extract, cotton wool extract, or mushroom compost extract, although these did not appear to contain appreciable quantities of IAA.

The fungus grew fairly well on basal medium solidified with agar which had been purified to remove vitamins. Addition of thiamin or biotin did not promote growth. Concentrations of IAA of 10 and 100 mg/l almost completely prevented initial growth while concentrations of 0.001, 0.1, and 1.0 mg/l were without effect. However, after about 30 days vigorous growth commenced in the medium originally containing 10 mg/l IAA and proceeded at the same rate as in its absence. It seems probable that this was due to inactivation of the IAA by the small amount of mycelium formed.

It is suggested that IAA may play a part in the growth processes of this fungus similar to that in higher plants, although the possibility that some related substance such as tryptophane is required for growth must also be considered, particularly in view of the unidentified growth-promoting factor present in various extracts.

I. Introduction

In recent years it has become clear that indole-3-acetic acid or closely related substances such as 3-indolylacetonitrile are the most common native auxins in higher plants (Larsen 1951; Jones *et al.* 1952). However, indole-3-acetic acid (abbreviated to IAA in the remainder of this paper) was identified as the auxin produced by fungi (Thimann 1935) considerably before its occurrence in higher plants was established.

On the other hand the relation of this substance to the growth of the fungi is still by no means clear as compared with our knowledge of its importance

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in the growth processes of the higher plants. Went and Thimann (1937) have reviewed earlier work in this field while Richards (1949) gives an account of some subsequent investigations. Probably the most striking evidence for a promoting effect of IAA on fungal growth is that provided by Kerl (1937), who reported that low concentrations of this substance produced a several-fold increase in the rate of radial spread and a more than 10-fold increase of fruit-body formation by *Pyronema confluens*. The most extensive study of the effect of IAA and several synthetic auxins on the growth of the fungi has been carried out by Richards (1949) who made a detailed investigation of the growth rates in solid medium cultures of four common fungi representing the four major systematic groups. With concentrations of between 0.0001M and 0.001M, she observed increases in growth rates at various stages of the order of 10 per cent. with all the fungi except *Schizophyllum commune*, the representative of the Basidiomycetes. The percentage spore germination of *Aspergillus candidus* was increased by low concentrations (less than 0.001M) of naphthaleneacetic acid. Richards also observed inhibition of growth and reduction in percentage spore germination with higher concentrations (0.001-0.01M) of IAA and the other synthetic auxins. This is a common phenomenon reported by several other workers (Leonian and Lilly 1937; Brian 1949).

In the light of the foregoing it seemed worth while, in connection with an interest in the physiology of soil fungi and particularly mycorrhizal fungi, to investigate further the relation of IAA to fungal growth. The white-capped form of the common cultivated mushroom, *Psalliota hortensis* (Cooke) Lange forma *albida* Lange, was chosen as the experimental organism for several reasons:

(1) IAA might well be present in its normal habitat—composted horse manure (Kogl, Haagen-Smit, and Erxleben 1934; Hamence 1944)—thus allowing the survival of an organism requiring this substance for growth.

(2) The Basidiomycetes have been investigated but little in regard to their relationship to IAA.

(3) Treschow (1944), after extensive investigation, has developed a synthetic medium essentially optimal for the growth of the brown form of the cultivated mushroom (*Ps. hortensis* forma *avellanea*) and probably also for the white form. He found either biotin or thiamin necessary for the growth of the brown form and claimed that they were completely interchangeable — a rather unusual and puzzling situation also observed in a large number of other Basidiomycetes by Jennison (1950). However, in Jennison's experiments biotin was not quite as effective as thiamin. Robbins and Kavanagh (1938) by contrast, found that what they designate as *Agaricus campestris* (quite possibly this was a form of the cultivated mushroom *Ps. hortensis*) showed no thiamin deficiency and grew quite well on a synthetic medium. Treschow (1944) found compost extracts to possess a growth-promoting activity about equal to that of biotin or thiamin and attributed it to the presence of these. However, extracts of alfalfa and nettle leaves appeared to contain some substances possessing considerably greater growth-promoting activity.

II. Materials and Methods

The strain of the organism used in this present investigation was obtained from a bottle of commercial mushroom spawn purchased from Australian Mushroom Industries, Sydney. The mycelium was growing under sterile conditions on moist wheat grains. Subcultures were made on a malt yeast extract ("Marmite") agar medium in petri dishes. Stock cultures from these were kept on slopes of the same medium.

In order to ascertain beyond doubt the exact identity of the organism and to test its fertility, a mushroom compost heap was prepared in the usual commercial manner under the guidance of Mr. D. Appledorf, an experienced commercial grower of Wahroonga, Sydney. Mushroom beds prepared from this were inoculated with 3 by 3 cm squares of mycelium grown on malt "Marmite" agar medium. A good crop of mushrooms resulted.

The systematics of the cultivated mushroom have been adequately discussed by Treschow (1944). He points out that it is not a variety of *Psalliota* (*Agaricus*) *campestris*, as commonly supposed, but a form of Lange's (1939) *Ps. hortensis* (Cooke) Lange. The fruit bodies obtained made possible the identification of the fungus used in this investigation as the cultivated white-capped form, for their characteristics agreed completely with Lange's (1939) description of *Ps. hortensis* (Cooke) Lange forma *albida* Lange. Treschow (1944) supports the further revision of Schaffer and Moller (1938) in using the name *Ps. bispora* (Lange) Schaffer & Moller forma *albida* Lange. Most of his experiments were done with *Ps. bispora* (Lange) Schaffer & Moller forma *avellanea* Lange, which is the cultivated brown-capped form.

The optimal basal synthetic culture medium developed by Treschow (1944) was used in all the experiments reported here. It contained the following per litre:

Glucose	10 g	$CaCl_2$	0.2 g
Asparagine	1 g	$FeCl_3$	0.01 g
KCl	0.2 g	KH_2PO_4	0.14 g
$MgSO_4.7H_2O$	0.2 g	$Na_2HPO_4.12H_2O$	1.8 g.

Although Treschow considered the addition of trace elements unnecessary, the following were added: B, 0.01 p.p.m.; Cu, 0.1 p.p.m.; Mn, 0.02 p.p.m.; Mo, 0.02 p.p.m.; Zn, 2 p.p.m. (Ryan, Beadle, and Tatum 1943; and cf. Lindeberg 1944; Perlman 1949*a*, 1949*b*). All inorganic chemicals employed were of A.R. quality while the asparagine was a C.P. synthetic product. The glucose was C.P. quality further purified by double recrystallization from 80 per cent. ethanol. Double-glass-distilled water was used for all solutions.

All glassware was cleaned with hot, soapy water, followed by chromic acid. It was then rinsed six times with tap water and three times with double-glass-distilled water.

The medium was made up by following the procedure suggested by Treschow (1944) but, contrary to his statement, a certain amount of precipitation occurred. The precipitate was, however, readily dispersed evenly through the medium by stirring so that identical amounts were received by individual culture vessels. The medium was generally dispensed to the culture vessels before being fully diluted—usually at double strength. By addition of solutions of IAA, vitamins, etc., or water it was made up to the volume specified.

For solid medium 15 g/l of agar were added. Unless otherwise stated, this was a purified agar prepared by a slight modification of the methods already described in detail by several workers (Robbins and Ma 1941; Ryan, Beadle, and Tatum 1943). It involves thorough leaching with 5 per cent. aqueous pyridine, distilled water, and 95 per cent. ethanol. According to these investigators, this removes the traces of biotin, thiamin, pyridoxin, and hypoxanthine present in agar, as well as certain inhibitory substances. Traces of IAA would also be removed as this substance is readily soluble in ethanol and to a lesser extent in water.

The pH (determined with glass electrode) of this medium after autoclaving for 10 min at 120°C was generally 6.9 and always within the range 6.8-7.0. Treschow (1944) found pH 6.8 to be optimal for growth and stated that the medium had this pH. Slight variations in the time of heating are probably responsible for these insignificant differences. No appreciable changes in pH resulted from the addition of small amounts of various vitamins, IAA, or natural extracts. The pH had rarely changed more than 0.2 at the end of an experiment even when considerable growth had occurred.

The IAA and vitamins (thiamin hydrochloride, pyridoxin hydrochloride, calcium pantothenate, nicotinamide, riboflavin, *p*-aminobenzoic acid, and inositol) used were all in the form of pure synthetic crystalline solids except the biotin, which was in ampoules containing 25 μg dissolved in 1 ml of sterile distilled water (prepared by Ashe Laboratories Ltd., London).

Water extracts of mushroom compost were prepared from samples dried at 80 ± 3°C for 72 hr. The dried sample (100 g) was boiled under reflux with 500 ml of double-glass-distilled water for ½ hr. This was filtered off and the procedure repeated. The extract was autoclaved and diluted to 1 l before use. Extracts of cotton wool (Johnson & Johnson, "New Era") were prepared in the same way except that 900 ml of water was used in the first extraction and only 100 ml in the second. As a yeast extract, "Marmite" (Sanitarium Health Food Co., Sydney) was used, dissolved in double-glass-distilled water.

The inoculum was prepared in a manner similar to Treschow (1944). Third-generation subcultures on 15 ml of basal medium solidified with purified agar in 9 by 1.5 cm petri dishes were usually employed. For the most part 2-mm discs were cut from these with a sterile cutter. In certain experiments 4- or 5-mm discs cut from cultures growing on unpurified agar medium were used. Treschow (1944) found that the age of the culture and the position in the plate from which the discs were taken had no effect on the growth as measured by dry weight. These observations were confirmed in preliminary experiments on the growth on liquid basal medium. Inoculum used in the experiments here reported was from plates 20-40 days old and was cut from 5 to 10 mm behind the edge of the colony.

The growth responses of the organism to the various substances tested were mostly examined in liquid culture by determining the dry weight of the mycelial mat. Pyrex conical flasks (250 ml) containing 50 ml of medium were used as culture vessels. In order to protect the thiamin molecule from being split into its components by heating at the pH of the medium (Treschow 1944; Rosenburg 1942) all vitamins and "Marmite" were autoclaved in 5 ml of distilled water in small test tubes resting in a sloping position in the flask. After cooling, the solution was tipped into the medium in the flask which had been diluted to only 45 ml. The discs of inoculum were usually allowed to float on the surface of the medium (cf. Treschow 1944) but in some experiments were sunk to the bottom of the flask. All cultures were dry weighed after 28 days, this period being chosen for the reasons set out by Treschow (1944).

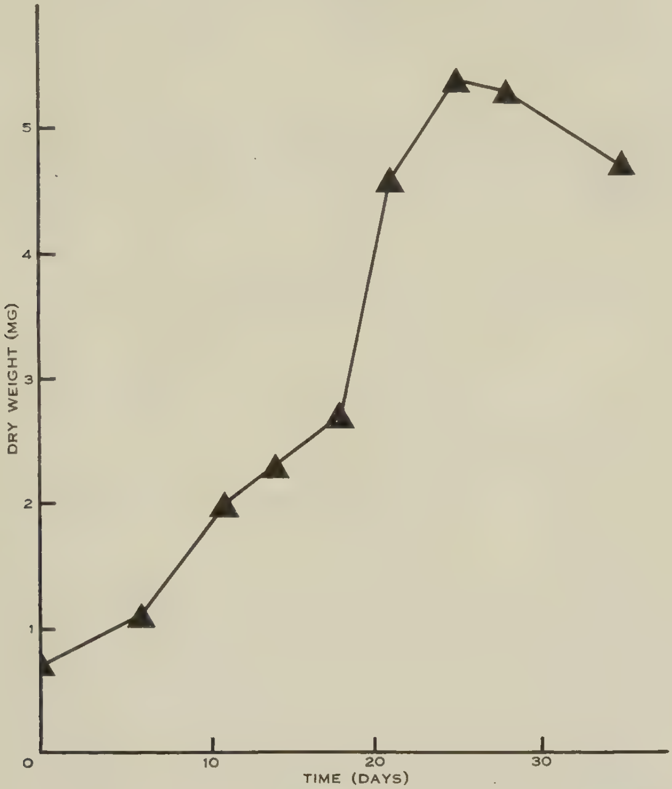

Fig. 1.—Time course of growth on liquid basal culture medium from 5-mm discs of inoculum cut from a culture on basal medium solidified with unpurified agar. Points shown are the mean dry weights of five replicates.

The colonies were filtered off on fine muslin which allowed any precipitate remaining in the medium to pass through with negligible loss of mycelium. After being transferred to weighing tubes the mycelium was dried at 100 ± 1°C for 24 hr and then weighed.

In a few experiments the growth responses were determined by measuring the diameters of cultures growing from a 4-mm disc of inoculum on 30 ml of solid medium in 9 by 1.5 cm petri dishes. Measurements along two marked diameters on each plate were made every few days. In a few cases the dry weight of the growth on solid medium was determined also. The agar was melted by plunging the petri dish into a large beaker of boiling water, thus freeing the mycelium (cf. Day 1942; Fries 1943), which was then treated as for liquid cultures. All cultures were grown at 24 ± 1°C, the optimal temperature according to Treschow (1944).

The results given are the mean of usually five replicates. In the tables of dry weights the standard error for each mean is also given. Since the dry weights obtained were rather variable within any given series, statistical methods were employed to determine whether differences were significant. The *t*- and *F*- tests as described by Snedecor (1946) were used for this purpose. Differences whose probability of chance occurrence was less than 0.05 were regarded as significant.

The IAA content of the various extracts and of the culture medium in certain experiments was determined using a slight modification (Ballard and Curtis 1948, unpublished data) of the standard *Avena* curvature method (Went and Thimann 1937).

TABLE 1

GROWTH ON LIQUID BASAL MEDIUM WITH VARIOUS INOCULA

Type of Inoculum	Dry Weights (mg)
5-mm disc unpurified agar	$4\cdot6\pm0\cdot6$
2-mm disc purified agar	$2\cdot0\pm0\cdot3$

III. RESULTS

(*a*) *Effect of Various Vitamins on Growth on Liquid Basal Culture Medium*

Very little growth took place on the liquid basal culture medium when a 5-mm disc from a culture on basal medium solidified with unpurified agar was used as inoculum (Table 1, Fig. 1). The results given in Figure 1 also show that it was not continuous and ceased after 25 days. This suggested that the organism was completely unable to synthesize one or more substances required for growth under these conditions (cf. Fries 1938, 1943; Lindeberg 1944). The slight growth which took place must be due to carryover of traces of growth substances in the inoculum or to traces of these in the medium, or both.

The use in most subsequent experiments (Tables 2-4) of a 2-mm disc of inoculum from a culture on basal medium solidified with purified agar significantly reduced this growth (Table 1). It was not considered worth while endeavouring to purify the medium further, the 2 mg of growth being negligible and quite satisfactory as a control for comparison of the effect of additions of various growth substances.

In view of the probable existence of a growth substance deficiency, a number of vitamins were tested for their ability to compensate for this. Since Treschow (1944) observed that either biotin or thiamin markedly promoted growth, both these substances were tried in the amounts he claimed produced maximum growth (0.05 μg biotin and 0.5 μg thiamin per flask). As can be seen from Table 2, neither of these substances alone gave any significant increase

TABLE 2

GROWTH ON LIQUID BASAL MEDIUM WITH VARIOUS ADDITIONS

Inoculum—2-mm disc from culture on purified agar basal medium. Bn = 0·05 μg biotin per flask; B1 = 0·5 μg thiamin per flask; A.V. = 10mμM of thiamin, pyridoxin, *p*-aminobenzoic acid, calcium pantothenate, nicotinamide, and riboflavin, 0·05 μg biotin, and 1 mg inositol per flask; A.V.—B2 = A.V. without riboflavin; IAA = 20 mg/l IAA

	Treatment	Dry Weights			
		Series *A* (mg)	Series *B* (mg)	Series *C* (mg)	Series *D* (mg)
1	Basal alone	2·4±1·0	3·9±0·5	2·0±0·3	2·1±0·2
2	Basal+Bn	1·6±0·5			
3	Basal+B1	8·3±2·6			
4	Basal+Bn+B1	10·3±2·3		9·9±2·7	8·3±1·6
5	Basal+A.V.—B2	11·4±1·7			
6	Basal+A.V.		14·5±0·7		
7	Basal+2 mg "Marmite" per flask	16·5±1·4			
8	Basal+20 mg "Marmite" per flask	77·5±18·6			
9	Basal+IAA	9·9±1·2			
10	Basal+Bn+IAA	9·1±0·5			
11	Basal+B1+IAA	34·4±9·6			
12	Basal+Bn+B1+IAA ..	42·2±15·5		60·3±27·8	40·8±10·0
13	Basal+Bn+B1+IAA 0·001 mg/l			5·6±0·7	
14	Basal+Bn+B1+IAA 0·1 mg/l			20·1±8·1	
15	Basal+A.V.—B2+IAA ..	55·0±19·6			
16	Basal+Bn+B1+IAA 25 mg/l			91·8±22·8	
17	Treatment 4. Series *A*, *C*, *D* pooled	9·4±1·2			
18	Treatment 12. Series *A*, *C*, *D* pooled	47·7±10·5			

in growth although thiamin may have exerted a slight promoting effect. A combination of these two gave a small but significant increase in growth. It was, however, a much smaller increase than that found by Treschow with either of them alone.

A mixture of eight vitamins in amounts of the order commonly used (cf. Robbins and Ma 1942*a*, 1942b)—10mμM of thiamin, pyridoxin, *p*-aminobenzoic acid, calcium pantothenate, nicotinamide, and riboflavin, 0.05 μg of biotin, and 1 mg of inositol per flask—was tested, but gave no significant further promotion of growth (Table 2). The omission of riboflavin was without effect. Since this mixture contained more than six times as much thiamin (10mμM = 3.37 μg) as was added previously, it was apparent that the lack of an appreciable effect of thiamin was not due to the amount supplied being too small. The same was probably true of biotin. It seemed that thiamin and biotin were the only substances with any effect, the former being the most important and possibly the only one effective.

As can be seen from Table 2, the effect of these vitamins was very small compared with that of small amounts of yeast extract ("Marmite"). It was apparent that some growth substance other than the above vitamins was present in "Marmite." It seemed possible that this could be IAA in view of the production of this substance by yeast cultures (Kogl and Kostermans 1934; Robinson and Stier 1941).

(*b*) *Effect of IAA on Growth on Liquid Basal Culture Medium*

The effect of IAA on growth on the liquid basal medium alone and in the presence of various vitamins was determined, the results being shown in Table 2. The concentration used most extensively here was 20 mg/l (1 mg per flask). This was chosen after preliminary experiments indicated growth promotion in the vicinity of this concentration and because it fell within the range commonly acting in a growth-promoting manner on other plant tissues under analogous experimental conditions (see Gautheret 1945; Mitchell, Burris, and Riker 1949 for summary).

It can be seen that on basal medium alone IAA brought about a small increase in dry weight production of the same order as that given by the combination of biotin and thiamin. The further addition of biotin (0.05 μg per flask) was without effect. In the presence of thiamin (0.5 μg per flask) there was a considerably greater though very variable increase in growth, this latter factor making the increase of doubtful significance. With the combination of biotin, thiamin, and IAA marked growth promotion occurred. It was again very variable, however, with the result that while in two experiments (treatment 12, series *D*; treatment 16) the increase was quite significant, in two other experiments (treatment 12, series *A* and *C*) it was quite possibly not significant. However, the data of three comparable experiments (treatments 4 and 12; series *A*, *C*, and *D*) could be pooled since they were carried out under identical conditions and an analysis of variance showed that they were not significantly different. The difference between the means of the two pooled treatments (17, 18) was then highly significant, indicating a considerable promotion of growth by the addition of IAA. In the presence of the vitamin mixture used previously (except for the omission of riboflavin, which sensitizes the photo-oxidation of IAA (Galston 1949; Galston and Baker 1949)), IAA again brought about a large but variable increase which was possibly not significant.

In an endeavour to secure better evidence for the growth promotion by IAA the concentration range of its activity was explored. Initially the two much lower concentrations of 0.001 and 0.1 mg/l were tested, the former giving no increase at all, while the latter gave an appreciable increase which, however, may not be significant (Table 2). It appeared that the growth-promoting effect was exerted mainly in the vicinity of 20 mg/l.

Accordingly, a range of concentrations in the vicinity of this was tested, the results being shown in the second column of Table 3. There was considerable growth promotion by all concentrations of IAA, there being no sharp optimum point within the range investigated. The same marked variability within a given series observed previously was shown with all concentrations. There may have been a decrease in growth promotion with 100 mg/l but the variability of replicates makes this doubtful. The growth promotion almost appeared to be of an all or none type although there may have been a range of increasing promotion below 5 mg/l. A preliminary experiment, the data of which have not been included, suggested that this was not the case, however. Further experiments are necessary, although the great variation between replicates may make it difficult to obtain any more satisfactory results.

TABLE 3

GROWTH OF FLOATING AND SUNKEN INOCULUM ON LIQUID BASAL MEDIUM WITH A RANGE OF CONCENTRATIONS OF IAA

Inoculum—2-mm disc from culture on purified agar basal medium. Bn = 0·05 μg biotin per flask; B1 = 0·5 μg thiamin per flask

Treatment	Dry Weights	
	Floating Inoculum (mg)	Sunken Inoculum (mg)
Basal+Bn+B1 alone	9·9±2·7	7·0±0·2
Basal+Bn+B1+5 mg/l IAA	57·8±32·6	8·6±1·8
Basal+Bn+B1+10 mg/l IAA ..	67·4±29·6	12·6±2·9
Basal+Bn+B1+13·3 mg/l IAA ..	68·9±24·7	10·7±1·9
Basal+Bn+B1+16·6 mg/l IAA ..	44·7±21·4	7·4±0·8
Basal+Bn+B1+20 mg/l IAA ..	60·3±27·8	9·0±0·9
Basal+Bn+B1+25 mg/l IAA ..	91·8±22·8	6·2±1·0
Basal+Bn+B1+30 mg/l IAA ..	66·2±22·9	5·1±0·2
Basal+Bn+B1+40 mg/l IAA ..	57·0±24·1	6·4±2·5
Basal+Bn+B1+50 mg/l IAA ..	86·8±30·3	8·6±1·7
Basal+Bn+B1+70 mg/l IAA ..	97·9±32·2	6·4±1·5
Basal+Bn+B1+100 mg/l IAA ..	36·5±19·1	4·9±0·6

An interesting aspect of the relationship of IAA to the growth of this organism was revealed by an experiment in which the inoculating discs were sunk to the bottom of the flasks instead of allowing them to float on the surface of the medium. The results of this experiment are shown in the third column of Table 3. The IAA did not appear to exert any appreciable growth-promoting effect under these conditions. It is noteworthy in this connection that the growth from floating inoculum discs in the presence of IAA was mostly of a fluffy aerial type with little or no development of submerged colonies.

(c) Effect of Certain Extracts on Growth on Liquid Basal Culture Medium

(i) *Yeast Extract, "Marmite".*—The growth-promoting effect with floating inoculum of small amounts of "Marmite" is shown in Table 2 and has been referred to previously. It is noteworthy that, while the inoculum was floating, considerable development of submerged colonies took place and there was considerable submerged growth from the inoculating disc. The growth was thus rather different from that with IAA as the growth-promoting substance.

The "Marmite" was found to contain approximately 21.5 per cent. moisture so that only 15.7 mg of dry matter were being added per flask or 314 mg/l. This was of the same order as the amount of asparagine or mineral salts added but since they were probably present in approximately optimal amounts (Treschow 1944) it seems likely that some other growth substance, possibly active and present in smaller amounts, was contained in the "Marmite." This was probably not identical with the eight vitamins tested. In an endeavour to ascertain whether this might be IAA, *Avena* assays (Went and Thimann 1937) were carried out on "Marmite" solutions of the same concentration as that present in the culture flasks and with several dilutions of this. No auxin activity could be detected.

Table 4

GROWTH ON LIQUID BASAL MEDIUM WITH VARIOUS ADDITIONS

Inoculum—2-mm disc from culture on purified agar basal medium. Bn = 0·05 μg biotin per flask; B_1 = 0·5 μg thiamin per flask; A.V.—B_2, as for Table 2; C.E. = compost extract; C.W.E. = cotton wool extract

	Treatment	Dry Weights (mg)
1	Basal alone (treatments 1-4)	2·4±1·0
2	Basal +A.V.—B_2	11·4±1·7
3	Basal+0·5 ml C.E. per flask	22·7±4·3
4	Basal+2·0 ml C.E. per flask	82·2±9·5
5	Basal alone (treatments 5-10)	2·3±0·3
6	Basal+Bn+B_1	5·4±0·8
7	Basal+5 ml C.W.E. per flask	4·9±0·6
8	Basal+5 ml C.W.E. per flask +Bn+B_1	11·7±0·9
9	Basal+10 ml C.W.E. per flask	6·7±0·5
10	Basal+10 ml C.W.E. per flask+Bn+B_1	34·7±6·2

(ii) *Compost Extracts.*—Treschow (1944) found that small amounts of compost extracts exerted a growth-promoting effect equal to that of biotin or thiamin. An extract of fully prepared compost ready for inoculation was tested here for its growth-promoting effect with floating inoculum. The results are shown in Table 4.

It is apparent that the compost extract exerted a growth-promoting effect considerably greater than that of any of the vitamins tested. The growth was of the same type as that found with "Marmite." The extract contained 0.72 per cent. dry matter so that when 2 ml was added per flask, 14.4 mg dry matter was added per flask or 288 mg/l. As with "Marmite," it seems probable that the growth-promoting effect was due to some growth substance possibly active in small amounts and not identical with any of the vitamins tested, rather than being due to any increase in the amount of some macronutrient.

This extract at several dilutions possessed no auxin activity in the *Avena* test. Another extract of only half-prepared compost which possessed approximately the same growth-promoting activity as the former had auxin activity equivalent to approximately 50 μg/l of IAA, i.e. 0.1 μg per flask when 2 ml of extract was added per flask or 2.0 μg/l. Since such low concentrations of IAA were without growth-promoting effect, the presence of IAA in the extract could hardly be responsible for its growth-promoting action.

(iii) *Cotton Wool Extracts.*—Through the accidental wetting of a cotton wool stopper in the autoclave, it was found that a water extract of cotton wool possessed growth-promoting activity in the presence of biotin and thiamin. Robbins and Ma (1942*a*) detected the presence of biotin, thiamin, and pyridoxin in cotton wool in the same way. The growth-promoting activity of a water extract of cotton wool with sunken inoculum was investigated, the results being shown in Table 4.

It is apparent that in the presence of biotin and thiamin small amounts of cotton wool extract were capable of markedly promoting the submerged growth of the mycelium. In view of this, special precautions were taken to ensure that all cotton wool stoppers were covered and kept dry during autoclaving in all experiments reported in the foregoing.

Since the cotton wool extract contained 0.019 per cent. dry matter, only 1.9 mg of dry matter was added per flask when 10 ml of extract was added to each flask, giving a concentration of 38 mg/l. The extract at several dilutions possessed no auxin activity in the *Avena* test. Some growth factor, not identical with IAA or the vitamins tested and active in small amounts, must thus have been present in the cotton wool extracts. The presence of biotin and thiamin appeared to be necessary for it to exert an appreciable growth-promoting effect.

(*d*) *Effect of IAA on Growth on Solid Basal Culture Medium*

Several experiments have also been carried out in an endeavour to ascertain whether growth promotion by IAA could be observed in cultures on solid agar medium.

As will be seen from Figure 2 and Table 5, considerable growth occurred on plates of basal culture medium solidified with purified agar. The inoculum was a 4-mm disc from an inoculum culture on unpurified agar basal medium. Addition of a mixture of biotin and thiamin at the concentration (0.03 μg biotin and 0.3 μg thiamin in 30 ml) found by Treschow (1944) to give maximum growth in liquid culture produced no significant increase in growth rate, the growth curve corresponding essentially to that on basal medium alone shown in Figure 2. However, since no attempt was made to protect the thiamin from being split into its pyrimidine and thiazole components by autoclaving at pH 7, some of it at least may have been in an inactive form (Rosenburg 1942; Schopfer 1943). Growth with unpurified agar was considerably more rapid than with purified, the rates being respectively 2.9 and 1.7 mm/day. Some growth-promoting substance had thus been at least partially removed from the agar. Growth on malt "Marmite" agar is much more profuse and dense than on basal medium with unpurified agar, indicating the presence of another growth substance or more of the same one. This is probably not identical with biotin or thiamin.

The effect of IAA on this growth on the basal culture medium was tested using a series of concentrations—0.001, 0.1, 1, 10, 100 mg/l. Concentrations of 0.001, 0.1, and 1 mg/l were without effect on the growth in diameter of the cultures. The curves for these concentrations followed very closely that shown in Figure 2 for growth on the basal medium and have therefore been omitted for the sake of clarity.

Table 5

GROWTH AFTER 36 DAYS ON BASAL MEDIUM SOLIDIFIED WITH PURIFIED AGAR

Inoculum—4-mm disc from culture on unpurified agar basal medium

Treatment	Dry Weight (mg)
No IAA	54·3±7·7
IAA 10 mg/l	26·4±2·1

The curve for growth with 10 mg/l shown in Figure 2 revealed a rather different situation, however. There appeared to be a complete cessation or inhibition of growth for the period from 12 to 30 days. Further, the appearance of initial normal growth in the presence of IAA is misleading as the growth consisted of only a few irregular hyphae on and above the surface of the agar. These extended, however, to approximately the same diameter as that of the dense mat of mycelium produced in the absence of IAA. After about 12 days, growth of these hyphae ceased altogether. Growth was thus markedly inhibited in the presence of IAA at a concentration of 10 mg/l in contrast to the growth promotion produced by this concentration in floating liquid culture. After 16-18 days, mycelial growth similar to that with lower concentrations or in the absence of IAA recommenced from the inoculum and attained to the diameter of the initially produced hyphae after approximately 30 days. Thereafter it proceeded as in the absence of IAA, giving a linear growth curve parallel to the control. The mean rate of radial spread in the period from 36 to 60 days was 1.7 mm/day, which was the same as the mean rate in the control over the period from 6 to 44 days. Extrapolation back to the time axis gives the probable curve of growth after what may be regarded as recovery from inhibition.

Dry-weight figures obtained at 36 days after inoculation are given in Table 5. The considerable inhibition of growth even after this time is quite apparent. Statistical tests showed the difference to be quite significant.

In an endeavour to obtain some explanation for the recovery from inhibition the standard *Avena* assay (Went and Thimann 1937) was used to determine the concentration of IAA present after 35 days from inoculation in the agar immediately in front of the mycelium and at the edge of the plate. In the former position the concentration was 0.03 mg/l while in the latter it was 0.08 mg/l or greater. This seems to indicate that some of the IAA originally present (10 mg/l) had been removed and that the mycelium was in some way responsible for this. A second disc of inoculum placed on the plate at this time about

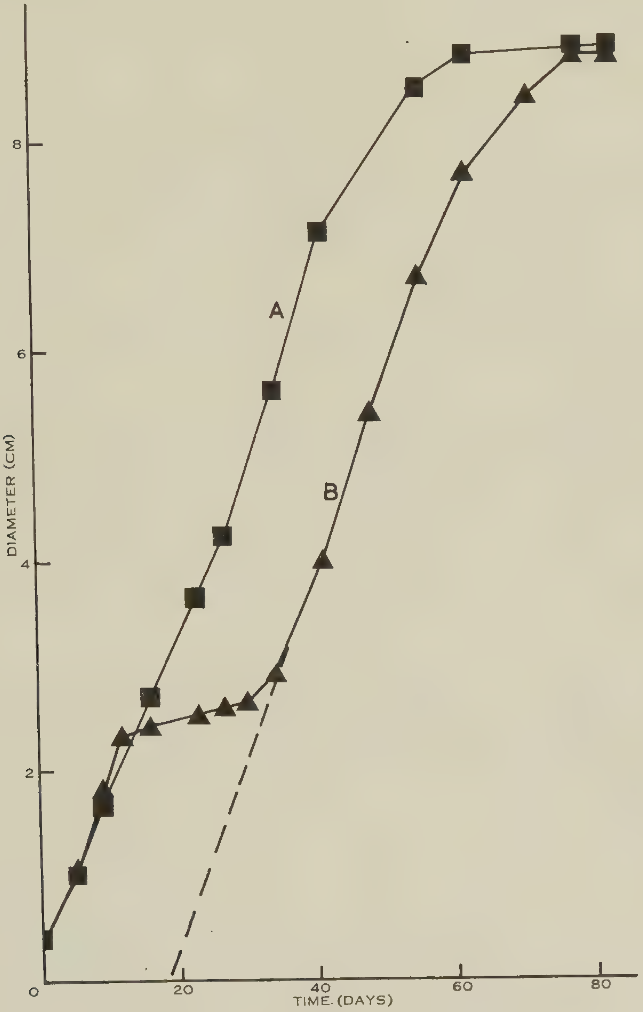

Fig. 2.—Time course of growth on basal medium solidified with purified agar without further addition (*A*) or with 10 mg/l IAA present (*B*). The inocula were 4-mm discs cut from a culture on basal medium solidified with unpurified agar. Points shown are the mean diameters of five replicates.

1 cm in front of the original growth grew quite normally, thus confirming that the concentration of IAA had been lowered beyond the inhibitory level at this point.

Only a few results were obtained for 100 mg/l IAA but it appeared that growth was initially similar to that with 10 mg/l. However, there was no recovery from the inhibition, such scanty growth as did occur ceasing after about 12-14 days, as with 10 mg/l IAA.

IV. Discussion

The vitamin requirements of this isolate of *Psalliota hortensis* appear to differ considerably from those of that used by Treschow (1944). It seems more probable that this is due to genetic differences in the strains employed rather than to such differences in cultural conditions as existed, e.g. culture at 24°C instead of the variable room temperature used by Treschow (1944). A number of such differences between strains in respect to vitamin requirements have been reported (Fries 1938, 1943; Robbins and Ma 1941, 1942*a*, 1942*b*; Lindeberg 1944).

The results presented show clearly that under certain rather precise conditions IAA was capable of markedly stimulating growth. While there was a definite growth promotion in floating liquid culture in the absence of any added vitamins, the presence of at least thiamin appears to be necessary for the maximum effect to be obtained. The concentrations of IAA effective in this growth promotion were of the same order as those found to have a growth-promoting effect on fungi by some other workers, notably Stuart and McLellan (1943), McLellan and Stuart (1947), and Richards (1949). These concentrations were also of the same order as those found to promote the growth and respiration of higher plant tissues such as stem sections and certain tissue cultures which appear unable to synthesize IAA (Went and Thimann 1937; Gautheret 1945; Mitchell, Burris, and Riker 1949). This suggests the possibility that the growth promotion by IAA may correspond to the similar phenomenon in higher plants.

The differences in the effect of the same concentration of IAA, e.g. 10 mg/l, on floating liquid, submerged liquid, and solid cultures are the most puzzling feature of the results. While a variety of factors influencing growth undoubtedly vary with these different experimental conditions, oxygen supply seems to be the one most likely to be significant. Thus a considerably decreased growth response to IAA by *Avena* coleoptile sections that are submerged in the solution compared with those that are floating has been attributed to differences in oxygen supply (Thimann and Bonner 1948, 1949; Bonner and Thimann 1950). Again, several workers (Nielsen 1928, 1930; Bonner 1932; Thimann and Dolk 1933) have shown that *Rhizopus suinus* produces IAA freely in solid and aerated liquid cultures but scarcely at all in non-aerated liquid cultures. Further experiments are planned to elucidate the possible relationship of these observations to the results obtained in this present investigation.

The inhibition of growth in solid culture by 10 and 100 mg/l IAA (0.002M and 0.02M respectively) parallels previous observations (Leonian and Lilly

1937; Brian 1949; Richards 1949) but little appears to be known about its mechanism. The recovery from inhibition could be attributed to the presence of an IAA oxidase similar to that known to occur in higher plant tissues (Larsen 1951).

The growth-promoting effect of compost, yeast, and cotton wool extracts is of considerable interest in relation to the effect of IAA. If it be supposed that the same growth factor was common to all, the data obtained with cotton wool extract suggest that the presence of thiamin and biotin is necessary for this factor to promote growth appreciably. It thus resembles IAA in this respect. Biotin and thiamin appear to have been practically absent from this sample of cotton wool in contrast to that used by Robbins and Ma (1942*a*). It seems probable that biotin and thiamin were present in "Marmite" (yeast extract) and the compost extract (cf. Bonner and Greene 1938; Robbins and Schmidt 1939).

The *Avena* assays indicate that the growth-promoting effect of the extracts cannot be attributed to a free IAA content of the order of the amounts shown to promote growth. Also the promotion of the growth of submerged cultures by the extracts factor suggests that it was not IAA. An interesting possibility is suggested by the known indole and tryptophane requirements of several other Hymenomycetes (Fries 1950*a*, 1950*b*). Either of these could be the extract factor. Under certain conditions IAA might be capable of supplying a requirement for them. However, they might equally well be converted to IAA and supply a requirement for this. A fairly extensive investigation now in progress will be necessary to determine the real situation.

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SALT UPTAKE BY ROOT TISSUE CYTOPLASM: THE RELATION BETWEEN UPTAKE AND EXTERNAL CONCENTRATION

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Summary

The proportion of a given length of bean root tissue which appears to reach the external concentration when placed in KCl solutions (the apparent free space, = A.F.S.) increases as the external concentration is increased.

The experimentally determined values of A.F.S. at various concentrations of the medium fit the curves representing the quantitative relations between A.F.S. and the external concentration deduced by assuming a Donnan equilibrium between the root cell cytoplasm and the medium at each concentration. Thus the experiments indicate the presence of non-mobile ions (probably anions) in the cytoplasmic phase. The concentration of these ions has been calculated to be of the order of 0.01 mol/l for both bean and maize root cytoplasm. Mature cells seem to have a higher concentration than those less mature.

The relation between uptake of KCl by bean root sections and the time of immersion has been found to follow Fick's law of diffusion. The apparent coefficient of diffusion for KCl in the tissue has been calculated. A typical figure is 0.07×10^{-5} cm ^{2}sec^{-1}, about one-twentieth of the value for an aqueous solution for the same concentration.

The results of these experiments are discussed in relation to the few existing evaluations by other investigators of the non-mobile ion content of cell cytoplasm.

I. Introduction

In previous papers (Hope 1951; Hope and Stevens 1952) it has been shown that uptake of salt by root tissue occurs in the first place by diffusion into the cell cytoplasm. The diffusion is reversible and is accompanied by diffusion potentials in which the cation/anion mobility ratio is greater than unity. About 8-12 per cent. of the weight of broad bean tips is penetrated by 0.01N KCl if it is assumed that this percentage of tissue reaches the same concentration as the medium. This diffusion process occupies 10-20 min and is followed by "active accumulation," in which a relatively constant amount of KCl leaves the medium every hour.

The present paper describes a study of the relation between apparent free space (A.F.S.) and the external salt concentration. A.F.S. is defined as the proportion of the tissue which appears to reach the external concentration during the initial uptake. The results show that the A.F.S. for the tissue, instead of being constant, as expected if KCl is diffusing from one simple aqueous phase to another, is a regular function of the external concentration and indeed is the function predicted by Donnan considerations; i.e. the results are consistent with the presence of a number of non-mobile ions in the bulk of the cytoplasm. The concentration of non-mobile ions (probably anions, from other considerations) has been estimated for various regions of bean roots and maize roots, and varies between 0.006 and 0.04M if the cytoplasm is considered simply as an aqueous phase.

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Part of the curve of uptake against time, when analysed, has been found to conform to Fick's law, thus giving additional evidence for supposing the initial uptake to occur by diffusion. By assuming reasonable values for the area and volume involved in the process, the apparent coefficient of diffusion for KCl in cytoplasm has been calculated.

Many writers (Blinks 1940; Frey-Wyssling 1948) consider plant cell cytoplasm to be a region containing immobile anions which are envisaged as electron excesses localized in the long-chain protein molecules which are constituents of the cytoplasm. However, there have been few attempts to find the concentration of these ions. Robertson (1951) says, "While the theory of Donnan effects resulting from cells in electrolyte solution is well developed, we are ignorant of the nature and concentrations of the substances on which ion exchange occurs." The only data available to the author are those of Meyer and Sievers (1936*a*, 1936*b*) and Meyer and Bernfeld (1946), who have analysed measurements of electric potential differences in various plant organs and conclude that many are consistent with the presence of a Donnan system in cytoplasm. Their "selectivity constant" when the solubility of the ions in the cytoplasmic phase is taken into account is a measure of the concentration of immobile ions, and this is of the order of 0.01M. The experiments to be described were undertaken at first in ignorance of the work of Meyer and his collaborators and thus provide an independent demonstration of the Donnan equilibrium in root tissue cytoplasm. Briggs and Robertson (1948) suggest a value of 0.1M for the non-diffusable ion concentration in carrot cell cytoplasm. This and their values for the coefficient of diffusion of KCl for the same tissue will be discussed in Section IV.

II. Material and Experimental Method

Broad bean (*Vivia faba* L.) roots were grown in sphagnum as described by Hope (1951). The experiments series *a* with bean roots, described in Section III, were made with a quantity of bean root tips 0.5 cm long containing the root cap, meristematic, and some elongating cells. Usually 40-50 tips, weighing altogether 0.5 g, were immersed in 10 ml of KCl solution to determine the uptake curve. Series *b* used the section 1 cm long between 2 and 3 cm from the root cap.

The maize roots were germinated on moist filter paper in large petri dishes in an incubator at 30°C. When 4-5 days old they were *c.* 6 cm long and at this stage the required section of the root was excised with minimum damage. The parts of the root used were the first, third, or fifth centimetre from the root apex for series *a, b,* and *c* respectively. In each series the excised sections were equilibrated in several changes of distilled water for 16-18 hr. At the end of this time the change of conductivity of the water containing the tissue was quite small (Hope and Stevens 1952).

Table 1

BEAN ROOT TISSUE—A.F.S. (PER CENT.)

Series *a*

Concentration	0·0023N	0·0034N	0·010N	0·035N	0·042N	0·055N	0·072N
Replicate 1	4·1	—	9·2	—	15·1	—	14·2
2	5·2	—	12·4	—	14·7	—	13·3
3	3·2	—	8·3	—	13·8	—	13·6
4	4·8	—	9·1	—	—	14·4	—
5	2·6	—	7·2	—	—	12·8	—
6	—	—	9·6	11·6	—	—	14·2
7	—	—	11·5	12·4	—	—	13·8
8	—	9·7	13·6	14·4	—	—	—
9	—	8·2	10·8	13·2	—	—	—
10	—	5·3	7·2	10·1	—	—	—
Means	4·0±1·0	7·7±1·8	9·9±2·0	12·3±1·4	14·5±0·6	13·6±0·8	13·8±0·4

Series *b*

Concentration	0·010N	0·034N	0·074N	0·100N
Replicate 1	4·7	8·9	—	13·9
2	4·0	11·8	13·2	15·3
3	2·5	11·2	12·7	13·5
4	4·0	10·8	12·8	14·6
5	2·7	7·8	10·7	12·9
Means	3·6±0·9	10·1±1·5	12·4±1·0	14·0±0·8

The uptake of electrolyte by the plant tissue was calculated from measurements of the conductivity of the medium in which the tissue was immersed. So long as cation exchange between the medium and cytoplasm has only a small effect on the conductivity of the former (see Section IV) the change in conductivity of a single univalent electrolyte gives the most accurate means of determining its change in concentration (see Robertson 1941). The sensitivity of the method has been improved so that changes in ion content of less than 10^{-7}M could be detected. This was done by using conductivity pipettes which had resistances of the order of $10^4\ \Omega$ in 0.01N KCl for experiments with the lower external concentrations, and pipettes which measured up to $2 \times 10^4\ \Omega$ in 0.01N KCl for the higher concentrations. The A.C. bridge was capable of measuring to within $\pm 1\ \Omega$ in 5000, using pipettes with freshly platinized electrodes. The change in conductivity of the medium during the initial uptake was usually less than 2 per cent. of the total conductivity so that the external concentration can be regarded as constant, as required in equation (**3**).

III. Results

(*a*) *Relation between A.F.S. in Excised Bean Root Sections and External KCl Concentration*

Table 1 gives the results of measuring the initial uptake of KCl and the A.F.S. calculated from this, for sections of broad bean roots in the increasing concentrations shown. Series *a* was made with the first 5 mm of the root apex excised from the rest of the seedling. Series *b* used the third centimetre of the root and thus included older but not fully matured cells. In every experiment the A.F.S. generally increased with increasing c_o (the external KCl concentration). The plot of average s (A.F.S. per cent.) against c_o in moles/l is given for these experiments in Figure 1. The full line is the theoretical relation between s and c_o derived as follows:

Table 2

Non-mobile anion concentrations and free spaces in bean root tissue

	A (mol/l)	$s_{\text{cyt.}}$ (%)	$s_{\text{res.}}$ (%)
Series *a* (First 5 mm from root apex)	0·006	14	0
Series *b* (Third cm from root apex)	0·04	15	0

If the region into which KCl is diffusing contains a constant concentration A mol/l of immobile ions (assume anions for this treatment, paired by K^+ ions only) then it can be shown by applying the Donnan distribution equations (assuming constant c_o and activities equal to concentrations) that the equilibrium concentration of KCl (c_i) in the cytoplasm is given by the following (see also Meyer and Sievers 1936*a*):

$$c_i = \frac{\sqrt{A^2+4c_o{}^2}-A}{2}. \quad \dots \quad \dots \quad \dots \quad (1)$$

Also, it is apparent that the A.F.S. takes the form

$$s = s_{\text{res.}} + s_{\text{cyt.}}\,(c_i/c_o), \quad \dots \quad \dots \quad \dots \quad (2)$$

where $s_{\text{res.}}$ is the residual A.F.S. due to intercellular and cellulose spaces and $s_{\text{cyt.}}$ is the percentage of the tissue occupied by cytoplasm.

I.e.

$$s = s_{\text{res.}} + s_{\text{cyt.}}\,\frac{\sqrt{A^2+4c_o^2}-A}{2c_o}. \quad \dots \quad \dots \quad (3)$$

For example, if $A=0$, $s=\text{constant}=s_{\text{res.}}+s_{\text{cyt.}}$ If A is not equal to 0, then s increases as c_o increases. The full lines in Figure 1 which pass close to all the average s values are the graphs of equation (3) with $s_{\text{res.}}$, $s_{\text{cyt.}}$, and A adjusted

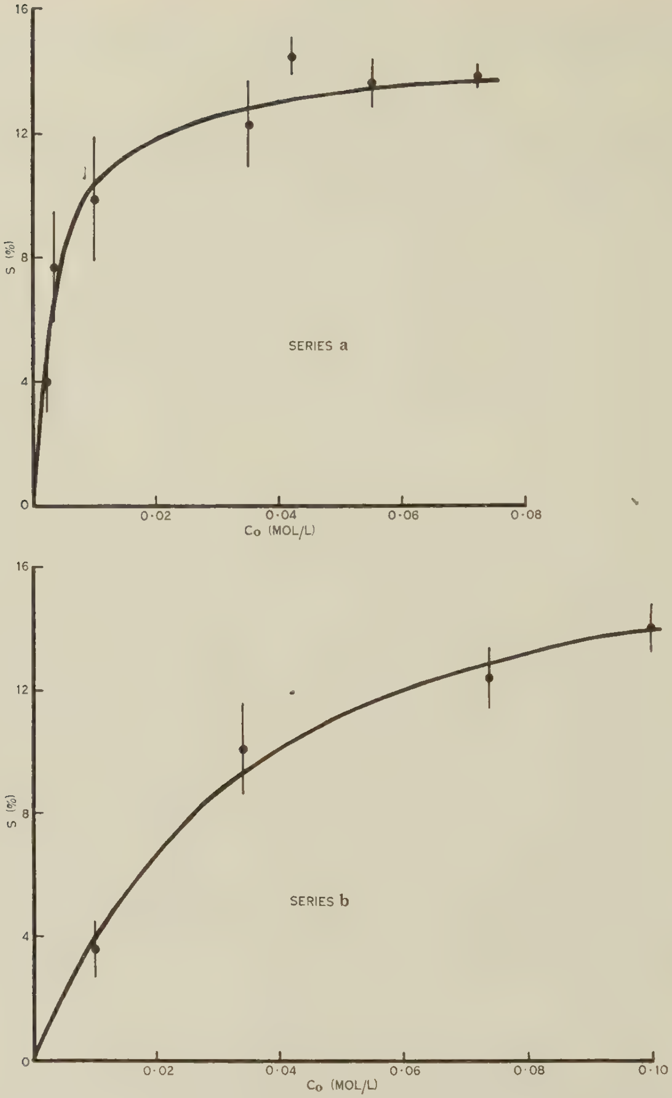

Fig. 1.—(*a*) Relation between apparent free space of bean root tissue, s (%), and concentration of c_0 of external KCl solutions. The tissue *a* was excised bean root pieces comprising the first 5 mm of root measured from the apex. The points plotted are the means and standard deviations for a number of replicates (see Table 1). The full line represents the theoretically deduced relation between s and c_0 derived from the theory of Donnan equilibrium as described in the text. In this tissue the non-mobile anion concentration A was approximately 0.006M and the cytoplasmic free space was 14 per cent.

(*b*) As above except that the experiments were with tissue *b*, the excised third centimetre of root measured from the apex. A was about 0.04M and $s_{\text{cyt.}}$ 15 per cent.

to give the best fit. Note that Y-axis intercept gives the value of $s_{res.}$, while the Y value approached asymptotically by the curve gives $s_{res.} + s_{cyt.}$. The values of these parameters for series *a* and *b* are given in Table 2. In Figure 1*a* and 1*b*, $s_{res.}$ is zero since the curves pass through zero at $c_o = 0$. Thus the free space due to non-cytoplasmic components of the tissue is negligible. It seems that in the root cells most of the A.F.S. is a phase containing immobile ions, the average concentration throughout the slice of tissue being of the order of 0.01M. *A* for series *b* was greater than that for *a*.

TABLE 3

MAIZE ROOT TISSUE—A.F.S. (%)

Series *a*

Concentration (N)	0·0049	0·010	0·029	0·070
Replicate 1	4·7	—	14·3	16·2
2	6·3	7·3	12·4	12·6
3	5·5	11·4	13·9	13·7
4	4·9	13·0	11·7	—
5	—	11·5	16·7	17·6
Means	5·4±0·6	10·8±2·1	13·8±1·7	15·0±2·0

Series *b*

Concentration (N)	0·010	0·030	0·056	0·070	0·084
Replicate 1	8·3	9·8	13·7	—	15·7
2	6·4	10·8	13·6	—	16·5
3	8·0	14·4	16·3	—	17·1
4	5·3	11·1	—	13·6	—
5	6·2	14·0	—	14·4	—
6	4·7	11·9	—	14·4	—
7	—	—	15·9	—	14·2
Means	6·5±1·3	12·0±1·7	14·9±1·2	14·1±0·4	15·9±1·1

Series *c*

Concentration (N)	0·010	0·030	0·072	0·104
Replicate 1	9·8	14·1	19·2	21·0
2	10·6	15·6	17·8	17·9
3	10·4	16·1	18·3	18·2
4	11·2	13·4	19·2	20·5
5	—	—	—	19·9
6	—	—	—	15·9
7	—	—	—	16·9
Means	10·5±0·5	14·8±1·1	18·6±0·6	18·6±1·8

In each replicate, the same tissue was placed in KCl of the given concentration for approximately 1 hr to obtain the uptake with time graph. In every case this took the general form shown in an earlier paper (first part of Figure 3, Hope and Stevens 1952). The tissue was then placed in several changes of distilled water for about 1 hr to wash out the KCl which had entered the free spaces. Further immersions in KCl of increased concentration and then distilled water etc. followed. Placing the root sections in most concentrations of KCl (up to 0.07N for series *a* and up to 0.10N for *b*) left them apparently undamaged. However, the cells began to 'leak' heavily after being in KCl of greater concentrations than given above and it is assumed that the permeability was increased irreversibly. Thus experiments were limited to less than or equal to these concentrations.

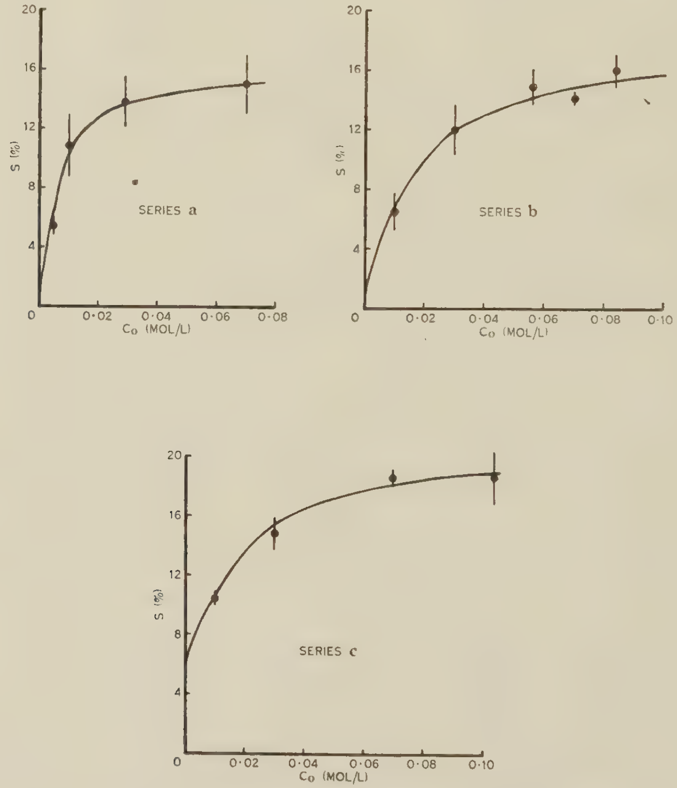

Fig. 2.—A.F.S. as a function of concentration of the medium for experiments with maize root pieces. *a*, *b*, and *c* represent separate experiments each with a number of pieces excised from young roots. The first, third, and fifth centimetre from the apex was used in experiments *a*, *b*, and *c* respectively (see Tables 3 and 4).

(*b*) *A.F.S. as a Function of* c_0 *for Sections of Maize Roots*

The above procedure was repeated using maize roots. Series *a*, *b*, and *c* in Table 3 represent experiments with the first, third, and fifth centimetres from the root apex, respectively, excised from the growing roots. The graphs of average A.F.S. against c_o are given in Figure 2. The curves of best fit, assuming a Donnan equilibrium at each external concentration, yield values of A, $s_{cyt.}$, and $s_{res.}$ as in Table 4. It is seen that the experimental points fit the theoretical curve well in each graph of Figure 2. A, the non-diffusable ion concentration, as with bean roots, is of the order of 0.01M. A increases with increasing distance from the root apex ($A_a < A_b < A_c$) and therefore on the whole it can be said that higher A is characteristic of more mature cells. It will be noticed that in Figure 2*c* the residual free space is not zero but about 6 per cent. This is probably due to the vascular tissue being more fully differentiated than in cells of *a* and *b*. Consequently the tissue contains a comparatively large volume of xylem which is filled with water after excision and acts as free space. The cytoplasmic free space $s_{cyt.}$ is approximately constant along the root—on the average 16 per cent.

TABLE 4

NON-MOBILE ANION CONCENTRATIONS AND FREE SPACES IN MAIZE ROOT TISSUE

	A (mol/l)	$S_{cyt.}$ (%)	$S_{res.}$ (%)
Series *a* (1st cm)	0·009	16	0
Series *b* (3rd cm)	0·02	17	0
Series *c* (5th cm)	0·03	15	6

(*c*) *Application of Fick's Law of Diffusion to the Uptake : Time Graphs*

If the entry of KCl into root cell cytoplasm is a process of diffusion, the driving force being a difference between the concentrations inside and outside the cytoplasm, the connection between the concentration c in this phase and time t should be as in Fick's law (see Davson 1943):

$$\mathrm{d}c/\mathrm{d}t = Da/v\left(\frac{c_i - c}{x}\right), \quad \ldots \quad \ldots \quad \ldots \quad (4)$$

where D is the coefficient of diffusion of KCl in cytoplasm, a the area through which KCl can diffuse, v the volume, and x the distance between regions of concentration c_i and c. Since the equilibrium concentration which is approached asymptotically is c_i, not c_o, because of the Donnan effect, the maximum difference in concentration at any time is $c_i - c$. On integration, if $c = 0$ at $t = 0$, and c_o (and therefore c_i) is a constant:

$$\ln \frac{c_i - c}{c_i} = -\frac{Da}{vx}t$$

or,

$$\log_{10} \frac{c_i - c}{c_i} = -\frac{Da}{2{\cdot}303vx}t. \quad \ldots \quad \ldots \quad (5)$$

Thus if the logarithm of $(c_i - c)/c_i$ is plotted against time, a straight line of slope $-Da/2.303vx$ should be obtained. From the uptake curve the average cytoplasmic concentration at any time t can be calculated since the cytoplasmic free space is known for a given length of root. Also, if $A = 0.04$M and $c_o = 0.103$N, $c_i = 0.085$N. The change in concentration caused by active accumulation can be allowed for. Plotting $\log_{10}(c_i - c)/c_i$ against time for a typical experiment with bean root tissue b in 0.103N KCl shows most of the points close to a straight line of slope -8.7×10^{-4} (Fig 3). This confirms the idea that the movement of KCl is by diffusion. The beginning of the graph has a greater slope, which is probably due to the high rate of uptake at the beginning while the concentration gradient is being established over the full distance from the surface of the tissue to the axis.

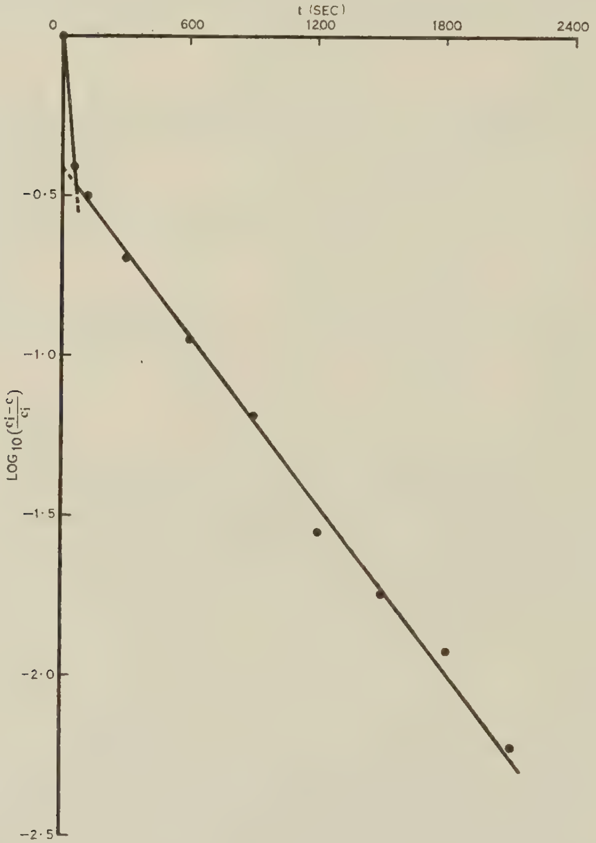

Fig. 3.—Plot of $\log_{10}$ $(c_i - c)/c_i$ against time t for a typical experiment in which the initial uptake of KCl by bean root tissue b in 0.103N solution was measured with time. Here c is the average cytoplasmic concentration at time t and c_i the equilibrium cytoplasmic concentration.

It is possible to calculate D (cm^{2}sec^{-1}) by making use of the identity between the slope of Figure 3 and $-Da/2.303vx$. However, this can be calculated only to an order of magnitude since the area through which KCl is diffusing cannot be estimated accurately. The *apparent* coefficient can be estimated as follows. The volume is put equal to $s_{cyt.}$, the cytoplasmic free space, since $s_{res.} = 0$ in the tissue. Thus v is about 15 per cent. of the tissue volume. The area is taken as equal to the surface area of the tissue (b) which is in the form of cylinders of average length 1.0 cm and average radius 0.11 cm. Actually the solute moves only through the cytoplasm of the roots and along wet cell walls. However, it is difficult to form an estimate of the area offered to the diffusing KCl by this part of the tissue. Using the value of v/a calculated on this basis and putting $x = 0.05$ cm (the measured uptake gives in turn the *average* cytoplasmic concentration and therefore that of the cytoplasm approx. half way to the axis), D is calculated to be 0.072×10^{-5}cm^{2}sec^{-1}. This is approximately one-twentieth of the value of D for 0.10N KCl at 20°C in aqueous solution, which is 1.67×10^{-5} cm^{2}sec^{-1} (International Critical Tables, Vol. 5). Other similar experiments with the bean roots gave the values 0.13, 0.094, 0.058×10^{-5} cm^{2}sec^{-1}. A value for the apparent coefficient of diffusion in the tissue much less than that for water could perhaps have been anticipated, having regard to the restricted diffusion paths. It should be noted that, as the vascular system in bean root tissue b is but slightly differentiated, diffusion into the root is practically all through the outer surface.

(*d*) *Conductivity Changes due to Water Movement from the Cell Vacuoles*

In view of the fact that the tonoplast in plant cells behaves as a semipermeable membrane, Mercer* (personal communication) has suggested that some of the change in conductivity observed in KCl solutions containing root tissue taken from distilled water may be due to water movement from the vacuoles to the medium. Because the cell walls are to a certain extent elastic, the cell volumes may follow changes in external osmotic pressure even though the vacuolar osmotic pressure is greater. (The osmotic concentration of plant cells is given by Bonner and Galston (1952) as 0.2-0.8M.)

To test this, some bean tissue b as used in (a) above was carefully weighed after (i) being taken from distilled water, (ii) the initial uptake from 0.08N KCl, and (iii) further washing for 30 min in changes of distilled water. If all the conductivity change is due to absorption of KCl from the medium, the weight should increase by up to 1 mg per g whereas if water movement causes all the change, the weight should decrease by about 13 per cent.

Table 5 shows the result of one such experiment. Before the tissue was weighed each time, excess liquid was mopped from its surface. In between the three weighings (see table) the tissue was moistened with the appropriate liquid and remopped before again weighing. It is seen that in this instance the mean decrease in weight was 0.029 g in *c.* 1.380 g, or about 2 per cent. Now the expected decrease in conductivity during the initial uptake, calculated from previous experiments, would have been 1.8 per cent. The appearance of 0.029 g water from the vacuoles in the medium (volume 10 ml) would cause a conductivity decrease of about 0.29 per cent. Thus the effect of the postulated water movement is small but not negligible. The figures (per cent.) for the A.F.S. of this tissue in the higher concentrations (Table 1) should be reduced by up to 2. However, the errors for concentrations less than this should become progressively smaller. The errors with bean tissue *a* are approximately the same, as shown by similar experiments to that described above.

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TABLE 5

WEIGHT (G) OF BEAN ROOT TISSUE *b* AFTER DIFFERENT TREATMENTS

	(i) Taken from Distilled Water	(ii) After 20 Min in 0·08N KCl	(iii) After Further 30 Min in Distilled Water	Mean Decrease in Weight of (ii)
	1·3740	1·3555	1·3925	
	1·3745	1·3460	1·3765	
	1·3735	1·3525	1·3860	
Mean	1·3740	1·3510	1·3850	0·029

IV. DISCUSSION

There have been many suggestions that plant cell cytoplasm may contain large 'multivalent anions' (Frey-Wyssling 1948) because of ionized groups on the long-chain protein molecules. Though these molecules are capable of giving rise to either acidic or basic groups, at ordinary external pH's the acid dissociation predominates. There is some evidence from potential difference studies that at least the surface of root cell cytoplasm behaves as though it contained H^+ ions absorbed to fixed anions, when the root is in a dilute acid (Lundegardh 1940; Hope 1951). This double layer of ions Lundegardh held to be a membrane highly resistant to diffusion of electrolytes.

The experiments described in Section III, in which the A.F.S. of root sections is measured as a function of external concentration, are consistent with the hypothesis of cytoplasmic fixed anions. It was found that the proportion of tissue which appears to reach the external concentration is increased. Thus either a fixed amount of the tissue reaches a proportionally higher concentration as c_o is increased or an increasing amount of tissue comes to the same concentration as the medium. The former suggestion has been found to be the more probable as the experimentally determined relations between A.F.S. and c_o have been matched by equations deduced from considering the free space as a region of non-mobile anions. The true free space is then a constant but the A.F.S. varies because of the Donnan effect. The average concentration of the non-mobile ions in the cytoplasm of various lengths of bean and maize roots has been estimated to be between 0.006 and 0.04M. The figures can be regarded as accurate *averages* for all the cells of the tissue because of the large number of roots involved in the determination, as long as the correction for water movement is applied. However, this has been determined only for the concentration 0.08N for bean tissue *b*.

The conductivity method was stated in Section II as giving an accurate method of determining change of concentration since each ion in leaving the solution gives rise to a known change in conductivity. This is so as long as the ionic exchange between the cytoplasm and the electrolyte does not also change the conductivity. In the present experiments, the pH of the medium did not change appreciably in the initial uptake so many H^+ ions are not available for exchange with the external K^+ ions. Also the ion conductances of the exchanged cations (K^+, Ca^{++}, and Mg^{++} ions are probably the most common cell metallic cations) are not sufficiently different from that of K^+ to give a conductivity change of more than 10 per cent., on the average, of that given by the disappearance of a K^+ and Cl^- ion pair from the medium. Thus the effects of the two processes on the conductivity of the medium differ by an order of magnitude. In any case after the first immersion in KCl, the number of exchangeable K^+ ions in the cytoplasm available for the next higher concentration would have increased, reducing the change in conductivity due to exchange still more. The error in the determination of A.F.S. due to water movement from the vacuoles was considered in Section III (*d*) above. Small errors are inherent in the values of A, $s_{\text{cyt.}}$, and D because of this effect.

The non-mobile anion concentration deduced from these experiments is of the same order as that calculated by Meyer and Sievers (1936*a*, 1936*b*) and Meyer and Bernfeld (1946) from electric potential difference measurements. By analysing the change of p.d. with concentration (using a fixed ratio of concentrations on the two sides of the tissue) they deduced A and u/v, the cation/anion mobility ratio for the electrolyte in the tissue cytoplasm. For the skin of the apple (using Loeb and Beutner's (1912) data) $A = 0.08M$ and u/v for KCl in the skin $= 1.2$ (Meyer and Sievers 1936*b*). Using cells of the alga *Chara*, Meyer and Bernfeld (1946) calculated A to be between 0.016 and 0.03M in the exterior surface of the cytoplasmic layer and between 0.024 and 0.046M in the interior surface. For the exterior of *Nitella* cytoplasm A was between 0.05 and 0.15M.

Briggs and Robertson (1948) suggest the value 0.1M for the non-mobile anion concentration in carrot discs from a measurement of 43.5 mV p.d. between two sides of a carrot disc with a 0.01N KCl solution on one side positive to a 0.1N KCl solution on the other. This value is calculated from Teorell's (1935) equation on the assumption that the ratio of ion mobilities in the disc is the same as in water (i.e. unity). However, it can be calculated that if $u/v = 2$, the p.d. of 43.5 mV corresponds to $A \cong 0.05M$ while if $u/v = 3$, it corresponds to $A \cong 0.03M$. Values of u/v greater than unity were found for KCl in bean roots (Hope 1951) and are quite possible in carrot tissue.

It is not clear why p.d. measurements with bean roots have not shown the Donnan effect. Usually the relation between p.d. change and log concentration is linear, indicating a simple diffusion p.d., instead of obeying the Teorell equation. However, it may be that the non-mobile anion concentration is not high enough in the epidermal cells (it is 0.006M on the average for all the cells in tissue *a*). Osterhout (1936) mentions that Donnan potentials do not seem to be present in his experiments with the large single cells such as *Nitella* and *Valonia*. The conditions of these experiments seem much the same as those of Meyer and Bernfeld (1946), who found the high non-mobile anion concentration in *Nitella* given above.

In a paper recently to hand, Tendeloo, Vervelde, and Zwart Voorspuij (1944) have shown how data such as that in Lundegardh (1940) connecting salt concentration and interfacial p.d. can be interpreted as due to a Donnan distribution of ions between the root and medium. The discrepancy between this explanation and that given by Hope (1951), who favoured a diffusion mechanism, is probably due to a difference in experimental material. In an extension of this theory, Vervelde (1948) considers the presence in the root of weak acids of varying dissociation and concentration. He has obtained values of *A*, the non-mobile anion concentration, and its variation with pH, by comparing theoretical equations with experimental values of the interfacial p.d. At pH values comparable with those of the KCl solutions in the experiments of Sections III (*a*) and (*b*) of this paper (i.e. pH about 5.5) the *A* value calculated for barley roots is about 0.02M, for wheat roots 0.04M, very similar values to those obtained by the completely different technique using bean and maize roots, described above.

The presence of non-diffusable anions in root cell cytoplasm gives a qualitative explanation of the difference in ionic mobilities for KCl diffusing in cytoplasm, mentioned previously. Negative ions would have comparative difficulty in diffusing compared with cations which could move by a series of exchanges.

The form of the graph of uptake of KCl plotted against time has been found to follow Fick's law of diffusion. By assuming reasonable values for the constants involved, it is possible to calculate the apparent coefficient of diffusion for KCl in bean root cytoplasm (an average for all the cells involved). A typical value was 0.072×10^{-5} cm^{2}sec^{-1}. Briggs and Robertson (1948) give the average value *c.* 0.03×10^{-5} cm^{2}sec^{-1} for the diffusion coefficient of KCl through carrot root discs. The diffusion paths through this tissue are probably similar in principle to those in bean roots. The intercellular spaces in roots are largely air-filled (see Hope and Stevens 1952) and the remaining paths consist of cytoplasm and wet cell walls. If this is so in carrot tissue, the coefficient for carrot is reasonably consistent with the value found for KCl in bean root tissue.

V. ACKNOWLEDGMENTS

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ENZYMES OF *ASPERGILLUS ORYZAE*

III. THE SEQUENCE OF APPEARANCE AND SOME PROPERTIES OF THE ENZYMES LIBERATED DURING GROWTH

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Summary

When a strain of the mould *Aspergillus oryzae* is grown in surface culture on a protein-free sucrose-tartrate medium, enzymes are released into the culture medium in the following order: first, the carbohydrases sucrase and amylase together with phosphatase, second, the proteases and esterases, and third, catalase. Their liberation is accompanied by the appearance of protein nitrogen in the medium and loss in weight of the mycelium.

Evidence is presented for the presence of at least two proteases in addition to glycylglycine dipeptidase—one reduces the viscosity of gelatin very rapidly and is believed to act preferentially on the high-molecular-weight fractions of gelatin (the "viscometric protease") whereas the other degrades the lower molecular components of the gelatin more readily (the "gravimetric protease"). The pH activity curves for sucrase and amylase also indicate the presence of more than one component.

Inactivation of the various enzymes present during incubation at 40°C and at different pH values has been followed.

I. Introduction

The development of a simple liquid medium for the production of highly active proteolytic solutions by surface culture of *Aspergillus oryzae* (Maxwell 1952) has provided material for further investigations on the properties and purification of the proteases and associated enzymes. Previous research on fungal proteases, such as that on taka-diatase, has been concerned with the examination of aqueous extracts of bran cultures of moulds (for example, Lichenstein 1944) or of extracts of ground mould mycelium (Johnson 1934). Solutions prepared in this way contain more colloidal material than the culture filtrates used in the present investigations and they are therefore less amenable to fractionation and characterization studies.

This paper describes the range of enzymes detected, their sequence of appearance in the liquid medium, and some properties of individual enzymes as determined by examination of dialysed and concentrated culture filtrates.

II. Enzymes Detected

Proteases which rapidly reduce the viscosity of gelatin solutions and cause rapid digestion of the lower-molecular-weight components of gelatin have been detected in the culture filtrate. Esterase, phosphatase, amylase, sucrase, and

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catalase were also liberated from the mycelium in sufficient quantity for convenient estimation. Enzymes catalysing the liberation of inorganic phosphate from phytic acid and lecithin, various dipeptidases, lactase, maltase, salicinase, nuclease, and urease were also detected and, judging by the loss of strength of cellophane during 3-4 hr dialysis of concentrated culture filtrates, a cellulase may also be present, but attempts to demonstrate the production of reducing sugar from cellulose during incubation for several days at 40°C were unsuccessful.

The following enzymes, which include several previously reported for *A. oryzae* (see, for example, Tauber 1949) were not detected: tannase, glucose dehydrogenase, glucose oxidase, tyrosinase, polygalacturonase. No significant hydrolysis of gum arabic, gum acacia, or agar agar could be detected.

III. Materials and Methods

(*a*) *Preparation of Mould Enzymes*

In experiments designed to measure the rate of enzyme production in the culture medium, the mould was grown at 22°C in 2-l bottles, each with 300 ml medium containing 4.0 per cent. sucrose, 3.0 per cent. sodium potassium tartrate, 1.1 per cent. NH_4Cl, 0.3 per cent. K_2HPO_4, 0.05 per cent. $MgSO_4.7H_2O$, 0.002 per cent. $FeSO_4.7H_2O$, and 0.1 p.p.m. Zn, adjusted to pH 6.2,* and giving a volume : surface ratio of 1.24. In all other experiments the volume of medium used was reduced to 225 ml and the ratio thereby to 0.93 in order to improve the yield of enzyme per unit volume of medium (Maxwell 1952).

The effect of pH on loss of activity of various enzymes during storage was studied using the filtrate from 10-day-old cultures. The culture filtrate (20 l) at 2°C was precipitated with 40 l ethanol at −20°C and the precipitate was suspended in 500 ml water and dialysed with agitation for 1 hr against tap water. The liquid was centrifuged to remove insoluble salts, dialysed for a further period of 2 hr in a rotating dialyser (Lennox 1949), and finally diluted to 2 l.

In the pH-activity studies, a mixed enzyme powder was prepared by adding acetate buffer at pH 5.6 to the above enzyme concentrate to give a final concentration of 0.1M, cooling to 2°C, precipitating with 4 l ethanol at −20°C, centrifuging, and vacuum-drying the precipitate in 3-5 hr. In determining the pH-activity curves for the action of proteases on some substrates a freeze-dried preparation of a dialysed enzyme concentrate was also used, and in some experiments protease-rich crystals were employed (Crewther and Lennox 1950).

(*b*) *Estimation of Enzyme Activity*

(i) *Protease.*—Estimations of the viscometric protease activity were carried out by the gelatin viscosity reduction method (Lennox and Ellis 1945) using an incubation period of 90 sec. Digestion of the lower-molecular-weight components of gelatin was determined by the gravimetric protease method (Crewther

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* In some instances the concentrations of some of the constituents were varied slightly.

1952). The degradation of haemoglobin and ovalbumin in pH-activity experiments was measured by the method of Anson (1938), the recorded pH being that of the enzyme-substrate mixture.

(ii) *Peptidase*.—Samples (0.1 ml) were removed from a 0.1M solution of peptide containing the enzyme before and after 4 hr incubation at 40°C. After the addition of 1 ml of 90 per cent. acetone, containing 0.001 per cent. naphthyl red, the samples were titrated with 0.1N HCl in 90 per cent. ethanol from a 0.1 ml Rehberg microburette (Linderstrøm-Lang 1927).

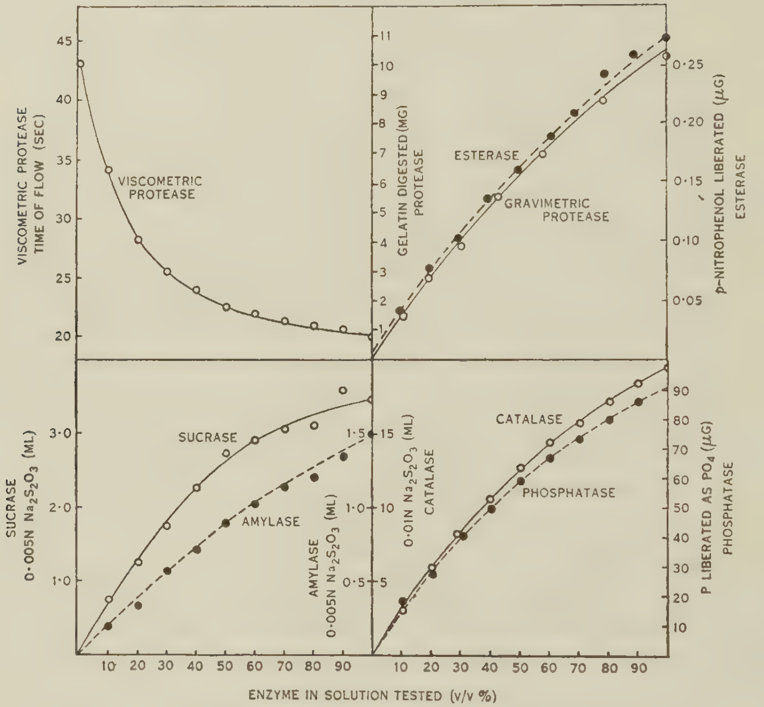

Fig. 1.—Dilution-activity curves of *A. oryzae* enzymes.

(iii) *Esterase*.—The method of Huggins and Lapides (1947) was used with *p*-nitrophenol acetate in phosphate buffer at pH 7.0 as substrate.

(iv) *Amylase*.—The reducing power of a mixture of 5 ml of buffer substrate (1.2 per cent. starch in 0.05M phosphate buffer at pH 7.5) and 1 ml of enzyme solution was estimated before and after incubation at 40°C for 1 hr using dilute Somogyi's reagent.*

(v) *Sucrase*.—As for amylase, but using a mixture of 1 ml of enzyme solution and 20 ml of 10 per cent. sucrose in 0.1N acetate buffer at pH 5.4, and incubating for 20 min at 40°C.

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* Prepared by mixing 2 volumes of Somogyi's reagent (Somogyi 1945) with 2 volumes 0.025N KIO_3 and 5 volumes distilled water, and filtering immediately before use.

(vi) *Phosphatase*.—Inorganic phosphate in a mixture of 3 ml of buffer substrate (0.25 per cent. sodium β-glycerophosphate, 0.25 per cent. $MgCl_2$, in 0.1M phthalate at pH 5.0) and 1 ml of enzyme solution was estimated before and after incubation at 40°C for 1 hr, by the method of Fiske and Subbarow (1925).

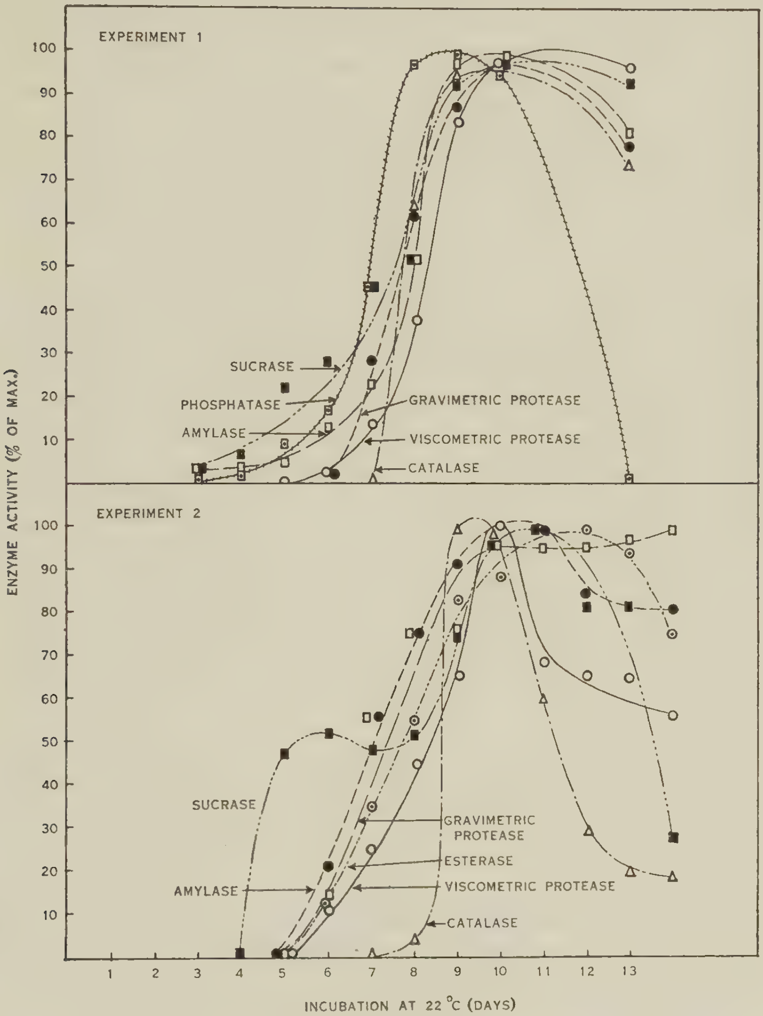

Fig. 2.—Enzyme liberation by *A. oryzae* at various stages of growth.

(vii) *Catalase*.—The buffer-substrate solution used contained 10 ml 100 volume H_2O_2 per 1 of pH 6.7, 0.01M phosphate buffer. After chilling to 0°C 0.5 ml enzyme was mixed with 2 ml substrate, the mixture was held for 30 min at 0°C, 2 ml 2N H_2SO_4 and 2 ml 10 per cent. KI were added, and the liberated I_2 was titrated with 0.01N $Na_2S_2O_3$.

A curve relating enzyme concentration to activity was obtained for each of the enzymes investigated (Fig. 1), and activity values were converted to rectilinear units of enzyme activity by reference to the appropriate curve.

(*c*) *Other Analytical Methods*

(i) *Weight of Mycelium.*—After filtration of the cultures, the tared filter paper and mycelium from each was dried for 7 days at 65°C and weighed.

(ii) *pH.*—pH was measured with a glass electrode assembly.

(iii) *Total Nitrogen.*—Estimated by H_2SO_4-H_2O_2 digestion of 1-ml portions of solution, containing 0.1-0.2 mg N, Nesslerization as directed by Koch and McMeekin (1924), and measurement of the light transmittance by these solutions at 412.5 mμ.

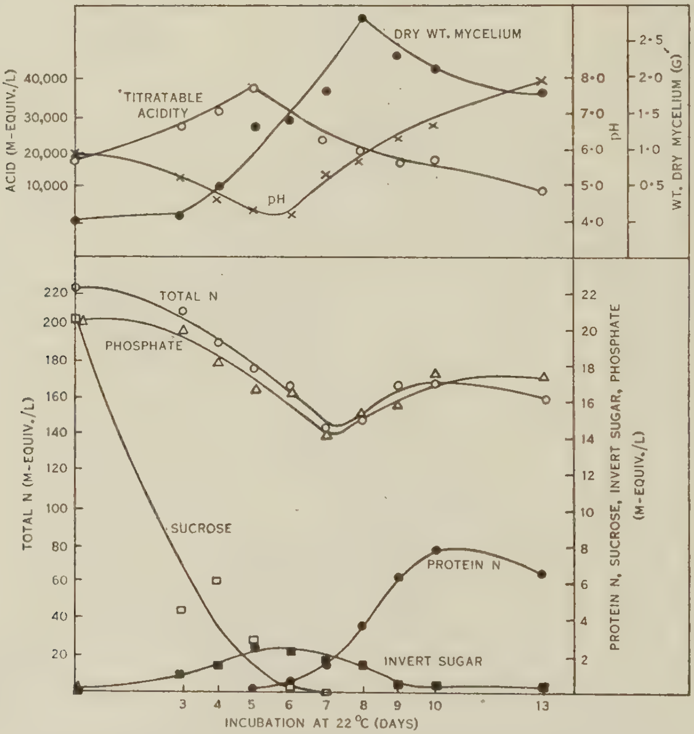

Fig. 3.—Changes in composition of medium during growth of *A. oryzae*.

(iv) *Precipitable Protein Nitrogen.*—After dialysis with continuous agitation for 4 hr, protein was precipitated from 1 ml culture filtrate by the addition of 2 ml 10 per cent. trichloroacetic acid, allowed to stand overnight to flocculate, centrifuged, washed with 10 per cent. trichloroacetic acid, and N in the precipitate estimated as described above.

(v) *Reducing Power*.—The apparent reducing sugar was estimated in 1-ml portions of the culture filtrate by the copper reduction method described above for amylase.

(vi) *Sucrose*.—The sucrose in 1 ml culture filtrate was hydrolysed by incubation for 24 hr at 40°C with 0.5 ml invertase (Pfanstiehl) and diluted to 20 ml for estimation, by the copper reduction method, of the increase in the content of reducing sugar due to the enzymic hydrolysis.

(vii) *Phosphate*.—The method used was that described for estimating the phosphate liberated by phosphatase.

IV. RESULTS

(*a*) *Order of Appearance of Enzymes and Associated Changes in Composition of the Culture Medium*

As will be seen from Figure 2, no enzymes were detected in the medium for several days after inoculation. Sucrase, phosphatase, and amylase were the first to appear after 4-5 days, followed by the proteases and esterase. Owing to the high sensitivity of the method of estimating catalase, traces of this enzyme were detected after only 3 days' incubation, but it was not until the eighth day that any significant fraction of the maximum catalase activity appeared. Other changes in the composition of the medium are shown in Figure 3. Of chief interest is the disappearance of most of the sucrose by the sixth day, the highest concentration of invert sugar occurring on the fifth day. This coincides with the appearance of significant quantities of enzymes and precipitable protein in the medium, both of which are maximal at the 10th day. The mycelial weight, on the other hand, is maximal at the eighth day with a rapid decrease to the 10th day corresponding with the rapid increase in enzyme activity.

TABLE 1

DISTRIBUTION OF VISCOMETRIC PROTEASE BETWEEN THE CULTURE MEDIUM AND THE MYCELIUM

Age of Culture (days)	Culture Filtrate (viscometric units)	Water Washings (viscometric units)	Water Extract (viscometric units)	$0 \cdot 1M$ $NaHCO_3$ Extract (viscometric units)
4	Nil	Nil	Nil	Nil
5	0·13	0·15	0·40	0·26
6	2·45	0·62	1·12	1·00

The amount of protease in the mycelium was determined by washing the mycelium with a volume of water equal to that of the culture medium (225 ml), the residuum then being extracted in the Waring Blendor with 225 ml of water followed by 225 ml of 0.1M $NaHCO_3$. The results for 5-day and 6-day mycelial mats are shown in Table 1.

(*b*) *Proteases of* A. oryzae

Considerable evidence has accumulated for the presence of more than one protease in the culture filtrates. For example, the ratio of the gravimetric protease activity to the viscometric activity, after surface culture of *A. oryzae* on a medium containing a mixture of acetate and formate in place of the usual tartrate, was found to increase from 5.8 to greater than 78 as the ammonium chloride content of the medium was reduced from 1 to 0.2 per cent. (Table 2). Citrate medium provided similar results.

Table 2

EFFECT OF AMMONIUM CHLORIDE CONCENTRATION IN THE MEDIUM ON THE RELATIVE AMOUNTS OF VISCOMETRIC AND GRAVIMETRIC PROTEASE PRODUCED

A. oryzae was grown on a medium containing 4 per cent. sucrose, 1 per cent. acetic acid, 0·5 per cent. formic acid, and salts as in the standard medium, and adjusted to pH 6·2

NH_4Cl in the Medium (%)	Protease Activity		Ratio
	Viscometric (arbitrary units)	Gravimetric (arbitrary units)	$\frac{\text{Gravimetric Activity}}{\text{Viscometric Activity}}$
0·2	1·6	125	78
0·5	1·1	33	30
1·0	1·3	7·5	6

The pH-activity curves obtained by the gravimetric and viscometric techniques also diverge to a considerable extent. The gravimetric technique (Fig. 4) suggests the presence of at least three enzymes with optima at pH about

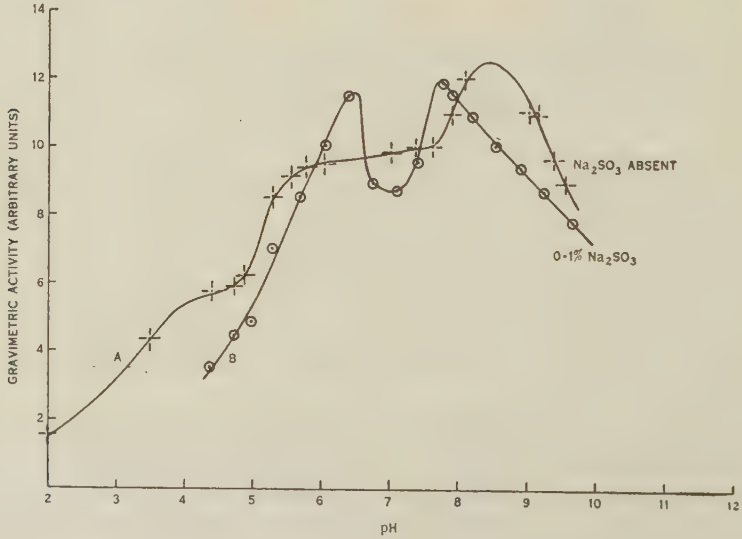

Fig. 4.—Effect of pH on the gravimetric protease activity.

4.5, 6.0, and 8.5, whereas the viscometric method, which also provides an atypical curve, suggests the presence of two components with optima at pH 7.0-7.5, and 10.0 (Fig. 5). Several enzymes also may be involved in the digestion of ovalbumin and haemoglobin.

TABLE 3

EFFECT OF INHIBITORS ON THE PROTEOLYTIC ACTION OF PARTLY PURIFIED MOULD PROTEASE

Inhibitor	Final Concentration (M)	Inhibition (%)	
		Viscometric Method	Gravimetric Method
Sodium *p*-aminophenyl arsenate	$1{\cdot}5 \times 10^{-1}$	27	Slight activation
Mercuric acetate	1×10^{-3}	67	Slight activation
Cupferron	1×10^{-3}	Slight activation	Slight activation
8-Hydroxyquinoline	1×10^{-3}	91	56
Iodoacetamide	5×10^{-1}	1	Nil
Sodium iodoacetate	5×10^{-1}	Nil	Nil

TABLE 4

EFFECT OF OXIDIZING AND REDUCING AGENTS ON THE PROTEOLYTIC ACTIVITY OF FILTRATES FROM TWO CULTURES OF *A. ORYZAE*

Enzyme Preparation	Reagent	Final Concentration of Reagent (M)	Protease Activity (% of original)	
			Viscometric	Gravimetric
Enzyme 1				
Undialysed	Water		100	100
	$KMnO_4$	$1{\cdot}5 \times 10^{-3}$	33	47
	I_2	$1{\cdot}5 \times 10^{-3}$	14	7
	H_2O_2	$3{\cdot}0 \times 10^{-2}$	65	245
Dialysed	Water		70	20
	Na_2SO_3	$1{\cdot}0 \times 10^{-1}$	40	95
	Cysteine	$2{\cdot}0 \times 10^{-2}$	80	40
	Cystine	$1{\cdot}0 \times 10^{-3}$	45	15
Enzyme 2				
Undialysed	Water		100	100
Dialysed	Water		90	73
	Na_2SO_3	$1{\cdot}0 \times 10^{-1}$	86	30
	H_2O_2	$2{\cdot}0 \times 10^{-1}$	94	0·3
	Na_2SO_3	1×10^{-1} (30 min after 2×10^{-1} H_2O_2)	88	16

The effects of sulphydryl-reacting compounds, metal chelating agents, oxidizing and reducing agents, and dialysis of the enzyme preparation have also been found to vary with the method used for estimating the protease activity (Tables 3 and 4). The presence of sulphite in the buffer substrate used for the gravimetric protease method also affects the shape of the pH-activity curve obtained by the gravimetric method (Fig. 4), the sulphite activating or inhibiting according to the pH. The effects of sulphite on the activity of the mould proteases also depend on the concentration of the enzyme used (Fig. 6).

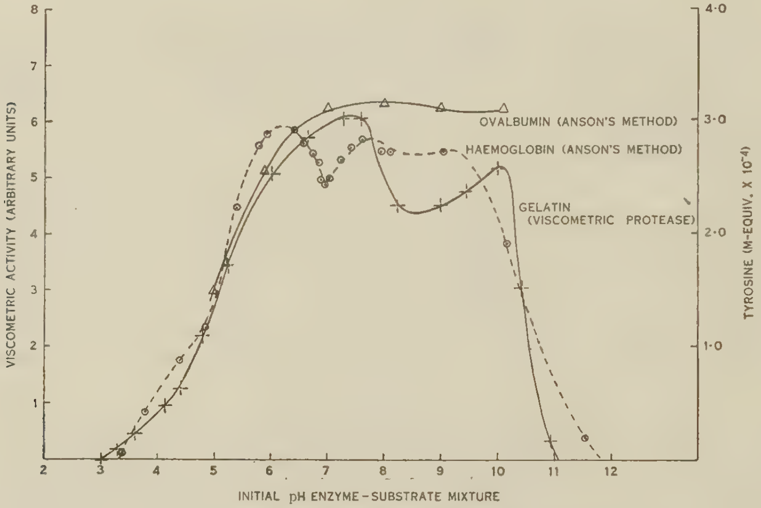

Fig. 5.—Effect of pH on the digestion of ovalbumin, haemoglobin, and gelatin.

The chemical evidence for the individuality of the proteases estimated by the two methods has been confirmed by physical experiments. A concentrate of the mould culture filtrate was fractionated with ethanol in 0.1M acetate buffer, and equal amounts of the fraction containing most of the proteolytic components were dissolved in aliquots of 0.01M pyridine acetate at various pH values. Cold ethanol was then added to 5-ml aliquots of the solutions in varying amount and the precipitates were dissolved in 50 ml water and proteolytic activity determined by both methods. As Table 5 shows, at pH 7.1 and 7.9 and in the presence of high ethanol concentrations, the precipitate obtained was rich in the gravimetric enzyme but contained little, if any, viscometric protease. It would be difficult to determine whether these results indicate that the two enzymes were separated or that one enzyme was inactivated by the procedures used, because the supernatant liquid from these experiments resisted attempts to recover the dissolved proteins by further addition of precipitating agents.

Filter paper electrophoresis of a freeze-dried, dialysed, mould culture filtrate was carried out. The paper was soaked in 0.02M glycine and about 0.12 ml of a 20 per cent. enzyme solution applied on a line across the paper from the edge of a well-cleaned razor blade. Electrophoresis was done at 1°C with a potential gradient of 9 V/cm for 6 hr, using the apparatus described by Woods and Gillespie (1953). Transverse strips of the paper, 1 cm wide, were then extracted with 4 ml of 0.05M $CaCl_2$, to prevent possible thermal inactivation of the enzyme due to the removal of cations (Gorini 1951), and the activities of the filtered extracts estimated by the viscometric and gravimetric techniques.

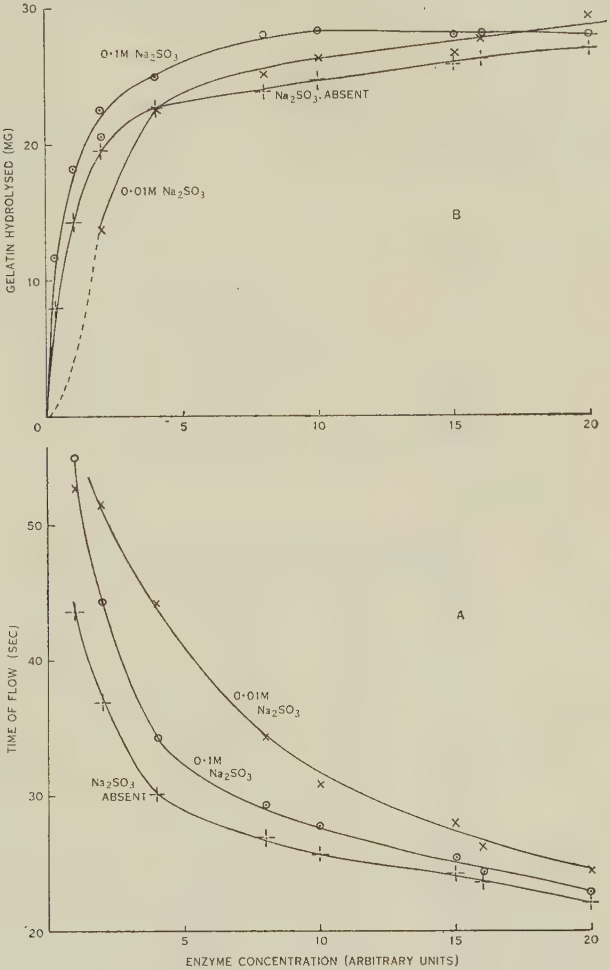

Fig. 6.—Inhibition and activation of protease by sulphite. *A*, inhibition of viscometric protease; *B*, inhibition and activation of gravimetric protease.

Care was taken that the enzyme solutions were not subjected to a long period at 40°C prior to estimation. The distribution of protease along the paper is shown in Figure 7, the gravimetric activities being plotted on a scale 1/1450 that of the viscometric figures. Within experimental error the distribution as shown by the two methods is identical, but whereas the ratio of activity as determined by the viscometric method to that determined by the gravimetric method was 1 : 1450 the ratio of the activities of the original material was 1 : 41. Paper electrophoresis experiments conducted in the above manner for periods of 0, 1, 2, 5, and 6 hr at 1°C each produced a single peak of activity but the total viscometric protease activity decreased progressively, the gravimetric activity remaining practically constant. That this inactivation was not due to removal of essential metal ions from the enzyme was shown by deionization of an enzyme preparation with a mixture of the "Amberlite" exchange resins IR, 4B, and IR 120, the final pH of the solution being 6.5. There was no change in the activity of the solutions. Similarly, electrodialysis of the enzyme solution did not appreciably affect the viscometric activity. The possibility that inactivation of the viscometric enzyme might result from separation of two enzymes essential for reduction of the viscosity of gelatin was eliminated by determining the activity of mixtures of the solutions obtained by extraction of the paper strips after electrophoresis. It could be further shown that, when enzyme solution was allowed to remain on paper containing buffer at pH 5.0 or 9.0 and the

Table 5

PRECIPITATION OF PROTEASE IN THE PRESENCE OF PYRIDINE

Solution of protease in 0·01M pyridine acetate precipitated with cold ethanol

pH	Ethanol (%)	Protease Activity of Solution of Precipitate	
		Viscometric Method (arbitrary units)	Gravimetric Method (arbitrary units)
4·0	44	Nil	Nil
	55	Nil	Nil
	62	Nil	Nil
	67	Nil	Nil
	71	Nil	4
6·0	44	Nil	Nil
	55	Nil	11
	62	0·4	91
	67	0·9	116
	71	0·6	75
7·1	44	Nil	2
	55	Nil	2
	62	0·4	71
	67	0·9	113
	71	Nil	119
7·9	44	Nil	1
	55	0·4	2
	62	1·0	72
	67	2·0	112
	71	0·5	120

whole of the enzyme recovered by extraction, there was a progressive loss in activity with time. Chromatographic experiments which separated the viscometric protease from the gravimetric protease in the enzyme concentrate were carried out in conjunction with Dr. H. Lindley. A suspension of the enzymes

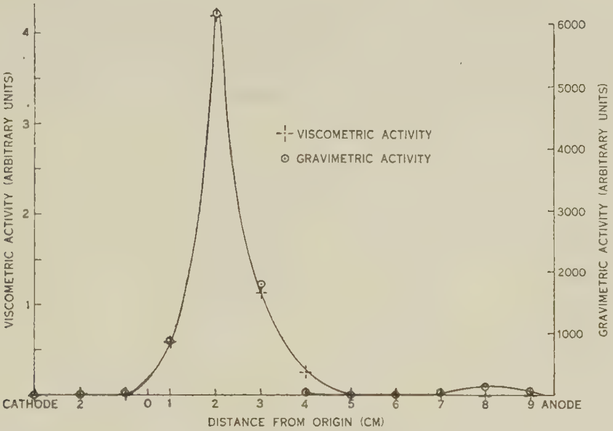

Fig. 7.—Distribution of proteases following paper electrophoresis of enzyme mixture.

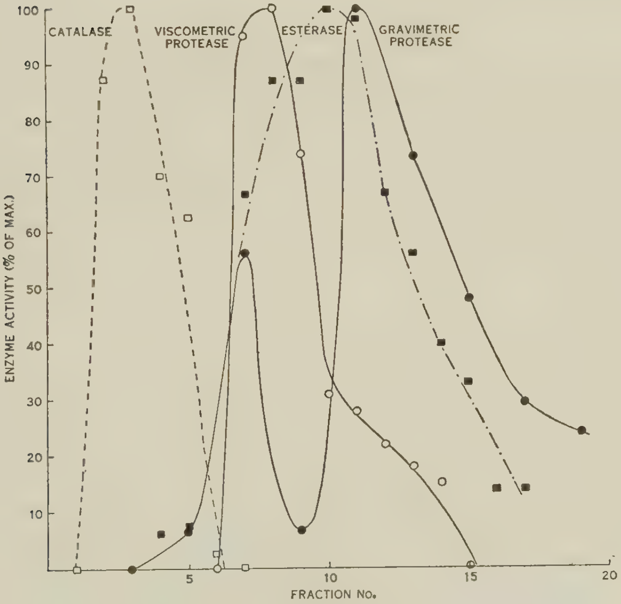

Fig. 8.—Chromatographic separation of some *A. oryzae* enzymes in ammonium sulphate solution on a silica column.

in 3M $(NH_4)_2SO_4$ (8 ml) was applied to a 3-g silica gel column and a solution of 2.5M $(NH_4)_2SO_4$ was used to develop the chromatogram. Successive 3.5-ml fractions were collected and the activity of various enzyme constituents was estimated. The distribution of activities is shown in Figure 8.

(*c*) *Hydrolysis of Glycylglycine and Other Peptides*

Of the available peptides which were soluble at 0.1M concentration, glycylglycine, L-leucylglycine, and DL-leucylglycylglycine were hydrolysed, the rate decreasing in that order (cf. the dipeptidase of *A. parasiticus* (Johnson and Peterson 1935)). Activation of glycylglycine hydrolysis by cobaltous ions in preference to other divalent cations, as shown for dipeptidases from various sources by Berger and Johnson (1939), has also been demonstrated for the glycylglycine dipeptidase of *A. oryzae*, the optimum concentration of cobalt ions being 0.001M (Figure 9). A shift of the optimum pH from 6.3 to 7.5 on addition of 0.001M cobalt sulphate (Fig. 10) is similar to that reported for peptidases by Berger and Johnson.

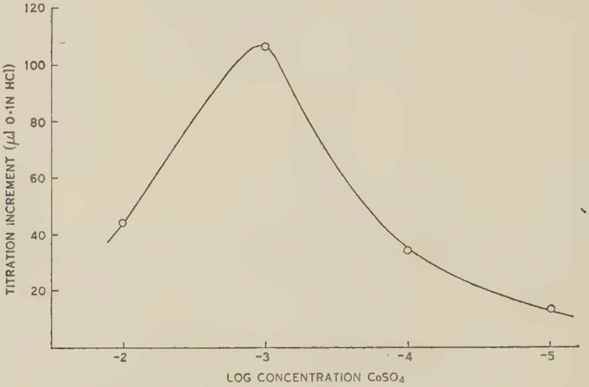

Fig. 9.—Effect of concentration of cobalt on activity of glycylglycine dipeptidase.

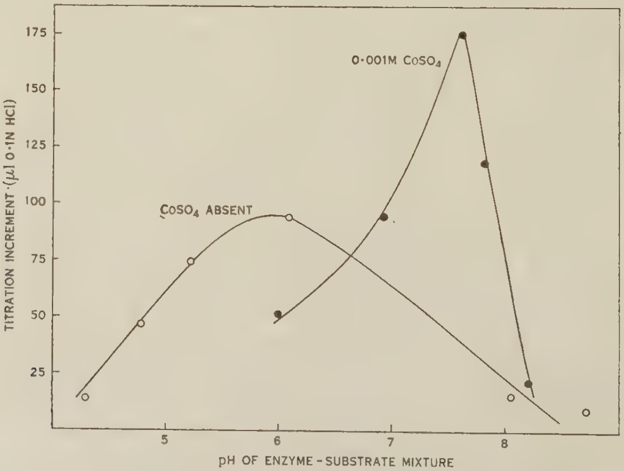

Fig. 10.—pH-activity curves for glycylglycine dipeptidase in the presence and in the absence of cobalt.

The glycylglycine dipeptidase is unstable both in concentrated solutions of the mould enzyme and in the protease-rich crystals under conditions which do not appreciably affect the activity of the other two proteases.

(*d*) *Effect of pH on Activity and Thermal Stability of Enzymes*

The pH curves in Figure 11 were determined using a solution of the mixed enzyme powder. The pH values are those of the enzyme-substrate mixtures measured immediately after incubation.

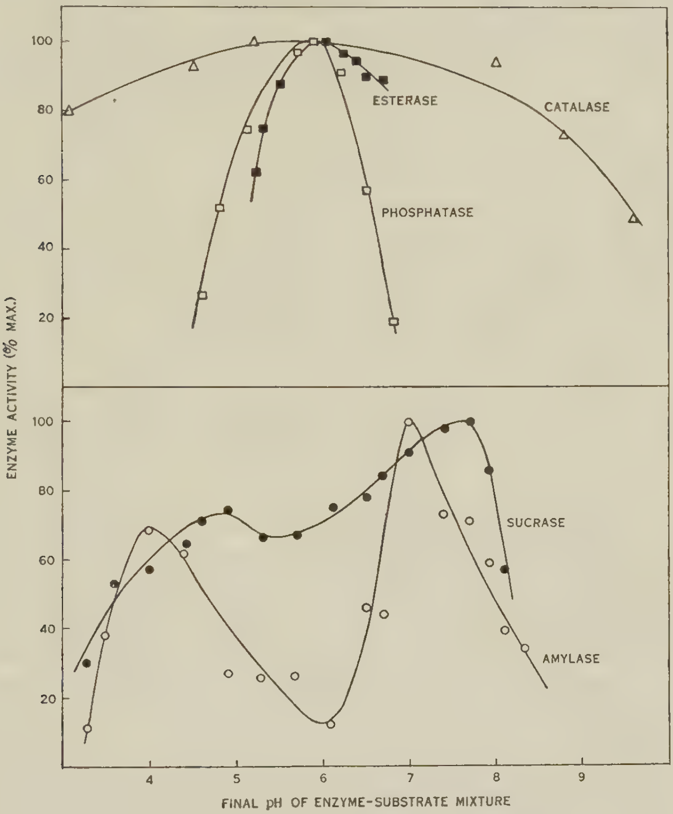

Fig. 11.—pH-activity curves for some non-proteolytic mould enzymes.

The thermal stability of the enzymes was measured by heating aliquots of unbuffered aqueous solution at pH 6.8 to various temperatures between 40 and 75°C. The resistance to heat was found to increase in the following order: catalase, phosphatase, esterase, amylase, viscometric protease, gravimetric protease, sucrase. The enzymes were found to have similar relative stabilities when heated as a suspension in 67 per cent. ethanol.

(*e*) *Effect of pH on the Digestion of Enzymes during Incubation*

During incubation of mixed enzyme concentrate for several days at 40°C without prior adjustment of the pH, catalase and phosphatase were almost completely inactivated in 1 day, but the other enzymes, particularly esterase, were more resistant.

The protein N precipitable with 2 volumes ethanol followed the protease and amylase activities closely (Fig. 12).

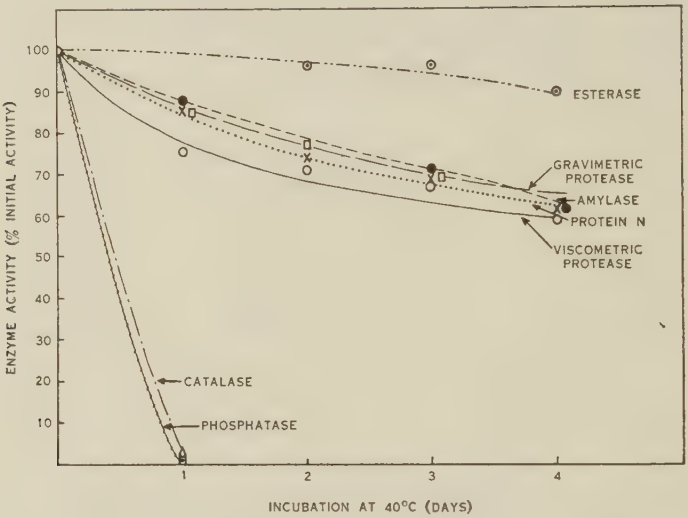

Fig. 12.—Fall in activity during incubation of mixed enzyme concentrate at 40°C.

The effect of pH on the disappearance of enzymic activity was investigated using a standard suspension of the enzymes prepared by vacuum concentrating the culture filtrate, precipitating with 67 per cent. ethanol, and storing at 2°C to prevent proteolysis. The precipitate was dissolved in 0.1M acetate buffer and held at the required pH for 17 hr at 40°C. After dialysis in the rotating dialyser (Lennox 1949) and adjustment to a constant volume, residual activities were determined (Fig. 13).

Fractionation of the concentrate with ethanol at pH 4.0 yielded four preparations, the first containing a high proportion of the catalase and little protease, the second containing mainly amylase and sucrase with little protease, and the third and fourth fractions consisting largely of protease and esterase (cf. Crewther and Lennox 1953). When portions of fraction 1 were incubated at 40°C with native and with heat-denatured fraction 3 inactivation of catalase was found to proceed much more rapidly in the presence of the native protease. Similarly the amylase and sucrase of fraction 2 were rapidly inactivated by the proteases of fraction 4.

The pH of optimum stability of the proteases has been found to depend on the conditions of the experiment. For example, addition of heat-denatured fractions 3 and 4 to fractions 1 and 2 respectively changed the pH of optimum stability from 5.5 to 5.0 in each case. On the other hand the proteases in a solution of crystalline material, containing 0.025 mg/ml, were most stable at pH 7.0.

V. Discussion

The results shown in Figures 2 and 3 indicate that the production of enzymes by *A. oryzae* in the culture medium is not directly associated with mycelial growth since there is no correlation between weight of mycelium and enzyme production. It may be significant that rapid release of enzymes into the medium occurred when the sucrose of the medium had been almost completely consumed and the invert sugar concentration was decreasing. Table 1 indicates that the mycelium can store a considerable amount of protease either as such or as a precursor which is converted to the protease on disruption of the cell structure. The decrease in mycelial weight from the eighth to the 13th day is probably due to release of stored enzymes into the medium.

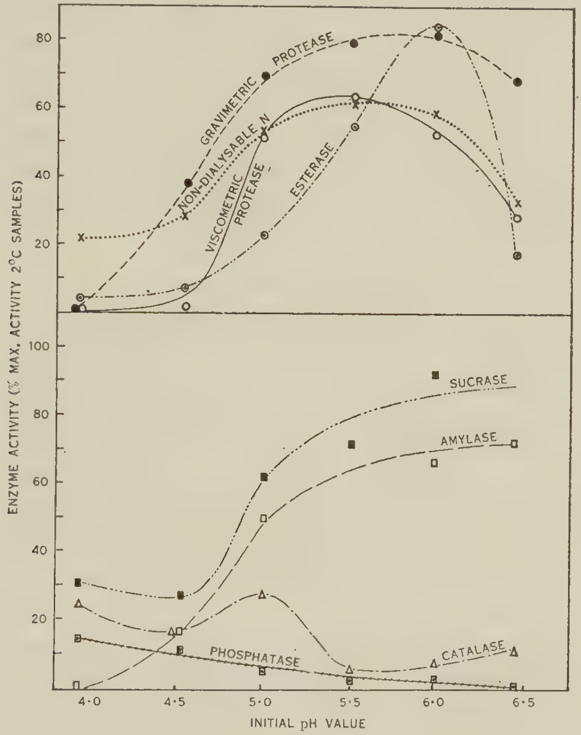

Fig. 13.—Effect of pH value on loss in activity after incubation for 17 hr at 40°C.

The evidence that the proteases estimated by the reduction in viscosity of gelatin and by the digestion of lower-molecular-weight components of gelatin in the gravimetric technique are different must be considered conclusive. Thus the ratio of the protease activity as measured by the two methods varies greatly according to the medium of growth, and also with the presence of sulphydryl-reacting substances, oxidizing or reducing agents, or metal chelating agents during activity measurement (Tables 2, 3, and 4). The pH-activity curves obtained with the two methods are dissimilar, and it has been possible to separate one of the enzymes almost completely from the other by the physical methods of ethanol fractionation, paper electrophoresis, or chromatography on silica gel. Figure 8 reveals the presence of two components attacking low-molecular-weight gelatin and also suggests that catalase and esterase should be separable from the other enzyme constituents.

The pH-activity curves of sucrase and amylase similarly indicate the presence of more than one component in each enzyme (Fig. 11).

The inactivation of the enzymes during storage in concentrated solution is apparently dependent on at least two factors, one of which is the protease activity of the solution. It is apparent that over the pH range 5.5-10.5 there is considerable protease activity, yet the proteases, amylase, and sucrase are most stable in the pH range 5.0-7.0. It has been shown that inactivation in the presence of active protease is much more rapid than in the presence of heat-inactivated protease, and furthermore the curve for non-dialysable nitrogen is similar to that for the stability of the proteases (Fig. 13). It is probable therefore that the enzymes are more susceptible to proteolytic attack when in a certain state of ionization. This state may correspond with the reversibly denatured form of enzymes postulated by Kunitz and Northrop (1934), or irreversible denaturation may take place readily at 40°C in certain pH ranges.

VI. ACKNOWLEDGMENTS

Grateful appreciation is due to Miss M. E. Maxwell who supplied the mould culture filtrate used in the experiments described in this and the two following papers in this series; also to Mr. J. M. Gillespie for providing the freeze-dried, dialysed preparation used in the paper electrophoresis experiments, to Dr. H. Lindley for permission to report unpublished data on the chromatographic separation of enzymes from mixtures, and to Dr. D. H. Simmonds for synthesizing and supplying some of the peptides employed as substrates.

Technical assistance with some aspects of the investigation by Mrs. H. M. Forss, Mrs. J. G. McIntyre, Miss C. M. M. MacKinnon, and Mr. D. N. Bleakley is also gratefully acknowledged.

One of the authors (F.G.L.) is indebted to Professor H. Raistrick for his interest in the early stages of the investigation and for generous hospitality during 1946-47 in the Biochemistry Department of the London School of Hygiene and Tropical Medicine.

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ENZYMES OF *ASPERGILLUS ORYZAE*

IV. FRACTIONATION AND PREPARATION OF CRYSTALS RICH IN PROTEASE

By W. G. Crewther* and F. G. Lennox*

[*Manuscript received December* 23, 1952]

Summary

Several methods have been examined for the separation of proteases from other enzymes and metabolic products in the culture filtrates obtained by surface culture of *Aspergillus oryzae* on sucrose-tartrate liquid medium. Concentration, by low-temperature precipitation with ethanol followed by ethanol fractionation of the dialysed concentrate at pH 4·5 in acetate buffer, is shown to be superior to adsorption methods or precipitation with flavianic acid or ammonium sulphate. At pH 4·5 the difference between the solubility of the proteases in aqueous ethanol solutions and that of other enzymes present is greater than at higher pH, and the addition of ethanol to the concentrate precipitates catalase, the carbohydrases amylase and sucrase, and finally the major proportion of the proteases and esterase, in that order.

Replacement of 0.1M sodium acetate by sodium valerate, ammonium chloride, and methylamine hydrochloride at the same concentration, produces minor changes in ethanol solubility of the various enzymes at pH 4. In the presence of glycine all the enzymes become completely soluble in 75 per cent. ethanol and in the presence of resonating aromatic amines, such as pyridine and aniline, the enzymes are almost completely soluble at all ethanol concentrations. By reducing the concentration of aromatic amine, precipitation of individual enzymes is possible.

By choosing suitable ethanol concentrations two fractions are obtained from the enzyme concentrate, the first containing catalase, sucrase, and amylase, with a little protease, and the second containing traces of sucrase and amylase with the greater proportion of the proteases and esterase. The addition of 4M ammonium sulphate to a concentrated solution of the second fraction results in the slow formation of needle-shaped crystals containing two proteases, glycylglycine dipeptidase and esterase, together with 70 per cent. ammonium sulphate. Recrystallization is most easily accomplished by cooling a saturated aqueous solution of the crystals.

I. Introduction

The previous studies on the enzymes of *Aspergillus oryzae* (Crewther and Lennox 1953) provide information concerning the nature, specificity, and stability of the various enzymes present in culture filtrates of the mould. Attention has now been turned to the fractionation of these enzymes with the primary object of purifying one or more of the proteases.

In this paper, further details of the optimal conditions for the formation of crystals containing the mould proteases (Crewther and Lennox 1950) are pro-

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vided, together with methods of recrystallization. The effects of pH and various anions and cations on the fractionation of the preparation with ethanol and methods of precipitation with other reagents are also described.

II. METHODS

The culture filtrates were prepared by surface culture of *A. oryzae* on sucrose-tartrate medium, and the estimation of enzyme activity, total N, and pH was made as described previously (Crewther and Lennox 1953).

Although the activities of many of the enzymes in the mould culture filtrate were high enough to be accurately measured, the total protein concentration, particularly in early batches, was too low for efficient fractionation with ethanol. Concentration of the culture filtrate was attempted firstly by adsorption. Of a number of adsorbents tested, only carbon black and calcium phosphate gel removed the viscometric protease from solution. Recovery of the enzyme by elution from the gel could be effected using buffer at pH 9.0 but it was not recoverable from the carbon black. The amount of calcium phosphate gel required was excessive and the method was discarded in favour of evaporation and precipitation methods.

Vacuum evaporation of 20-l batches of mould culture filtrate was carried out in a glass, climbing-film, cyclic evaporator having five heat-exchange tubes connected in parallel with a 5-l flask. A large-surface brass condenser was used, through which refrigerated water was circulated, and a low pressure was maintained by two water pumps and an oil pump protected by a dry-ice trap. After 4-5 hr the batch was reduced to 2 l, the temperature having risen from 25 to 35°C as concentration proceeded.

The concentrate obtained by evaporation was dialysed and stored at a low temperature. Proteolysis, with considerable loss of enzyme activity, was found to have occurred during the evaporation and, as more active culture filtrates became available due to modification of the method of cultivating the mould (Maxwell 1952), vacuum concentration was discarded in favour of concentration by ethanol precipitation.

The 20-l batch of culture filtrate was chilled to 2°C either by standing overnight at 2° or, more rapidly, by agitation in a stainless steel can in a freezing chamber at -25°C, and 40 l of ethanol at -25°C was added with agitation. During the addition of the first 10 l the temperature rose momentarily to 8°C but it was rapidly reduced to -2°C on further addition of ethanol. The flocculent white precipitate was allowed to settle for 1 hr in a cabinet at -25°C, most of the supernatant was decanted or removed by suction and discarded, and the precipitate was recovered from the remainder of the solution by centrifuging in an imperforate stainless steel basket. The precipitate was suspended in water and the suspension diluted to 500 ml.

The yield of viscometric protease in the concentrate obtained by this method has been found to depend largely on the extent of proteolysis during treatment. When the culture filtrate was held for 18 hr at 20°C prior to precipitation, the yield of protease in the concentrate decreased by more than 50 per cent.

The thick suspension of the precipitate was dialysed for 1 hr in 5-cm diameter cellophane dialysis tubing in a vessel through which water was flowing vigorously, the volume increasing to approximately 650 ml. Insoluble salts were then removed by centrifuging, and dialysis of the solution was continued for a further period of 2 hr in a rotating dialyser[*] (Lennox 1949), using 2.7-cm diameter dialysis tubing. Dialysis and other operations were carried out rapidly in order to minimize the formation by proteolysis of compounds which inhibit the final crystallization.

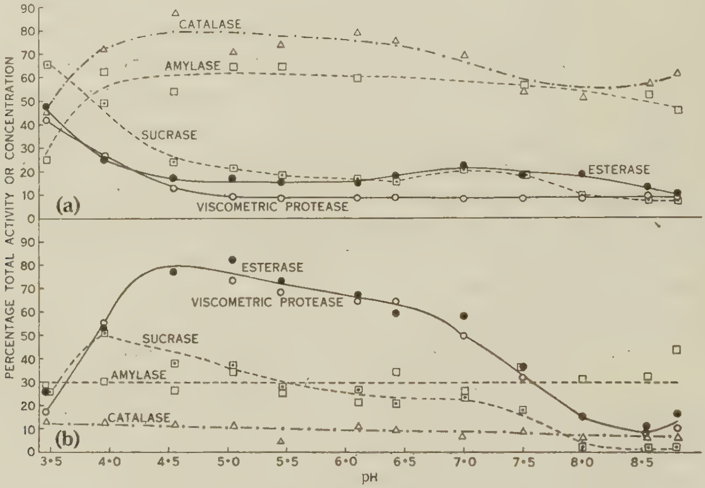

Fig. 1.—Activity of enzymes precipitated with ammonium sulphate at various pH values. (*a*) Precipitate by ammonium sulphate increment from 0 to 2.7M; (*b*) precipitate by ammonium sulphate increment from 2.7 to 3.6M.

III. Experimental

(*a*) *Precipitation of Enzymes with Ammonium Sulphate and Flavianic Acid*

Enzyme concentrate obtained by evaporation was mixed with 3 volumes of 3.5M ammonium sulphate containing suitable amounts of H_2SO_4 or NH_4OH and equilibrated for 18 hr at 18°C. The precipitates, when dissolved in 2 vol. of water, were found to have the activities shown in Figure 1(*a*). Addition to the supernatants of 4 vol. of 4M $(NH_4)_2SO_4$ having the same pH values produced a further series of precipitates which, when dissolved in 2 vol. water, yielded solutions with the activities shown in Figure 1(*b*). At the lower salt concentration catalase and amylase precipitated over a wide range of pH, whereas sucrase, esterase, and the viscometric protease precipitated maximally at pH 3.5, the minimum pH possible without considerable inactivation. With increase in salt concentration the sucrase, esterase, and protease precipitated at higher pH than previously and over a wide range of pH; the solutions at pH

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[*] Melbourne tap water contains *c.* 50 p.p.m. total solids, most of which is organic in nature.

3.5, having been partly depleted of these enzymes, did not produce very active precipitates.

Reduction of the pH of an enzyme concentrate to 4.0 by addition of flavianic acid caused precipitation of fine granules. When these were dissolved in 0.005N NaOH to give a pH of 7.0 and diluted to the original volume, a solution was obtained with proteolytic activity equal to that of the original. By shaking the active enzyme flavianate solution with "Amberlite" IR4 the yellow flavianate was completely removed without affecting the proteolytic activity. However, on adding greater amounts of flavianic acid and thereby reducing the pH to 3.5, a flaky precipitate formed which was devoid of activity.

The addition of 1.0M acetate buffer at pH 4.0 to mixed enzyme concentrate to reduce the pH value to 4.5 and fractionation by adding 10 equal increments of 1 per cent. flavianic acid did not result in clear-cut separation of the enzymes. Esterase and catalase were most active in fraction 5, gravimetric protease and amylase in fraction 6, viscometric protease and phosphatase in fraction 7, and sucrase in fraction 8. The total nitrogen content, determined after removal of the flavianic acid on "Amberlite" IR4, was maximal in fraction 6.

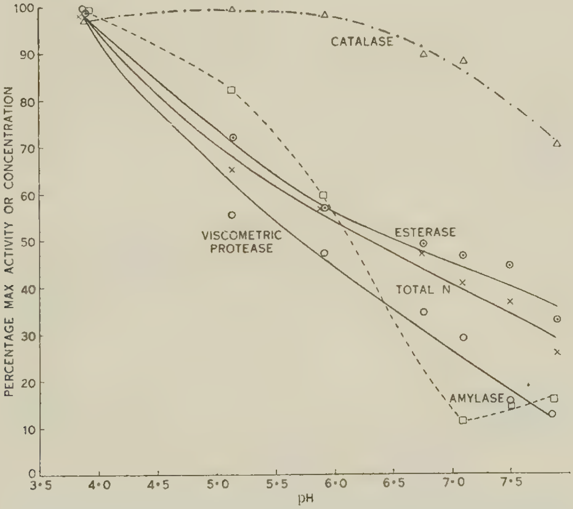

Fig. 2.—Activity of ethanol precipitates formed in presence of 0.05M phosphate buffers. Ethanol increment 0-56 per cent.

(*b*) *Effect of pH on Precipitation with Ethanol*

Figures 2-4 show the effects of pH on the ethanol precipitation of the various enzymes present in the concentrated filtrate using 0.05M phosphate, HCl in the absence of buffer, or 0.05M acetate to adjust the pH. An ethanol increment from 0 to 56 per cent. was tested for each buffer, and a further increment from 56 to 69 per cent. for concentrate adjusted with hydrochloric acid and acetate.

(*c*) *Precipitation with Ethanol in the Presence of Organic Ions*

The data presented in the preceding section suggest that the various enzymes in the concentrates exhibit greatest differences in their solubility in ethanol at low pH, and a pH of 4.0 has therefore been used to test the effects of different buffering ions at 0.1M concentration.

Mould culture filtrate, which had been concentrated under vacuum, was dialysed against running tap water, precipitated with two volumes of ethanol at 2°C, filtered, and the precipitate dissolved in a solution of the electrolyte being tested to give the original volume of filtrate at pH 4.0. Aliquots of this solution were then precipitated with various amounts of ethanol at 2°C and held at that temperature for 15 hr. Each precipitate was centrifuged off, dissolved in 10 vol. of water, and the solution used for the estimation of protease by both the gravimetric and viscometric methods. Catalase, esterase, sucrase, amylase, and total nitrogen were also estimated.

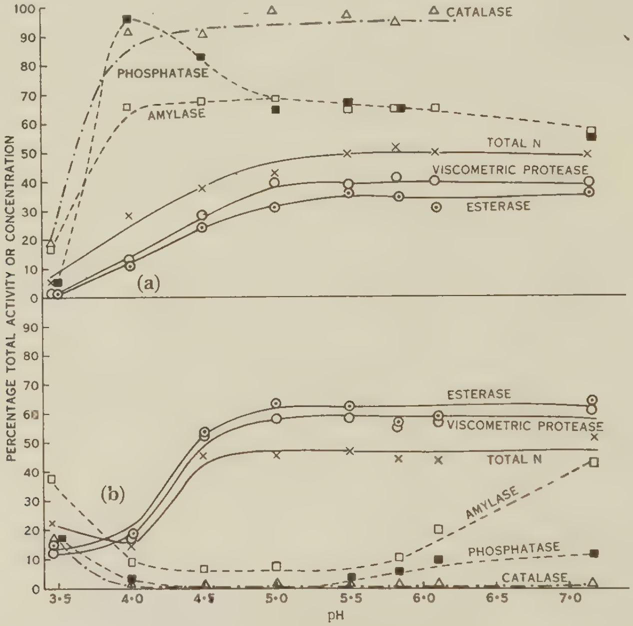

Fig. 3.—Activity of enzymes precipitated by ethanol after adjusting the pH with hydrochloric acid. (*a*) Precipitate by ethanol increment from 0 to 56 per cent.; (*b*) precipitate by ethanol increment from 56 to 69 per cent.

The changes which occur in precipitation of the enzymes when acetate is replaced by valerate are shown in Figure 5. The order of appearance of the enzymes in the precipitates is similar but the range of ethanol concentrations over which the enzymes precipitate is greater with valerate than with acetate. However, the slope of the curves relating ethanol concentrations to the amount of each enzyme precipitated is less with valerate than with acetate; there is therefore little to choose between the anions from the point of view of separation of the proteases from other enzymes.

When using glycine in place of acetate as the buffer (Fig. 6) the sequence of appearance of the enzymes and the ethanol concentrations required for precipitation of each enzyme were not markedly changed. It was found, however, that in 75 per cent. ethanol the enzymes became completely soluble or remained in the liquid phase as a slight opalescence which could not be centrifuged out.

Other amino acids used as buffers were lysine, δ-aminovaleric acid, α-amino-*n*-valeric acid, arginine, and cysteine hydrochloride. Their characteristics compared with acetate as buffer are summarized in Table 1. Unlike glycine, no appreciable tendency to increased solubility at high concentrations of ethanol was observed with other amino acids.

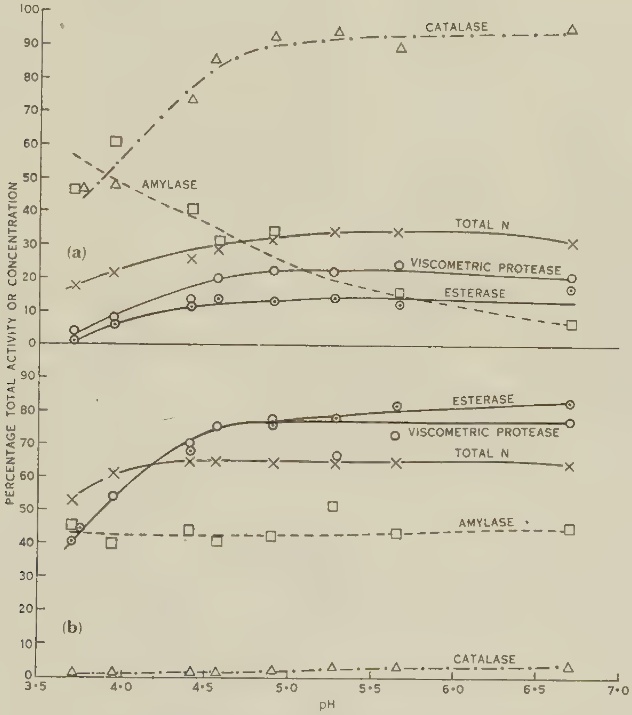

Fig. 4.—Activity of enzymes precipitated by ethanol in presence of 0.05M acetate buffers. (*a*) Precipitate by ethanol increment from 0 to 56 per cent.; (*b*) precipitate by ethanol increment from 56 to 69 per cent.

A number of simple amines have also been used under the same conditions as the anions and amino acids. As a basis for comparison, ammonium chloride was first tested and provided the results shown in Figure 7. Comparing it with acetate the most noteworthy difference is to be found in the tendency for catalase, sucrase, and amylase to precipitate at higher ethanol concentrations. A second difference is to be found in the delayed appearance of esterase in the precipitates. The results for methylamine (Fig. 7), like those for di- and trimethylamine, were similar to the curves obtained with ammonium chloride.

Tetramethyl ammonium bromide provided curves which were almost identical with those given by ammonium chloride.

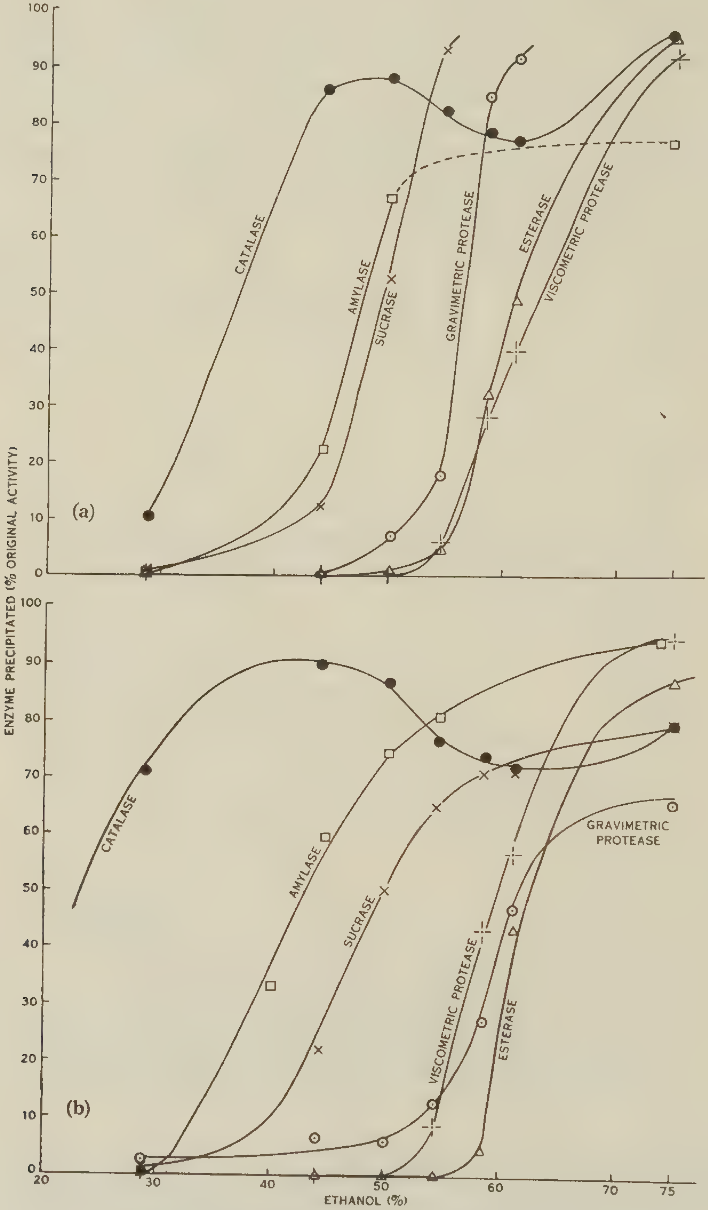

Fig. 5.—Ethanol precipitation curves in presence of 0.1M acetate (*a*) and valerate (*b*) buffers at pH 4.0.

Entirely different results were obtained when the aromatic amines were tested. With 0.1M aniline or pyridine at pH 4.0 no precipitate was obtained at any of the ethanol concentrations tested. In the presence of *p*-phenylenediamine and 28 per cent. ethanol, a slight precipitate was obtained which contained about 30 per cent. of the total catalase but no other enzyme. At higher concentrations of ethanol no precipitate was obtained. As shown in Figure 8,

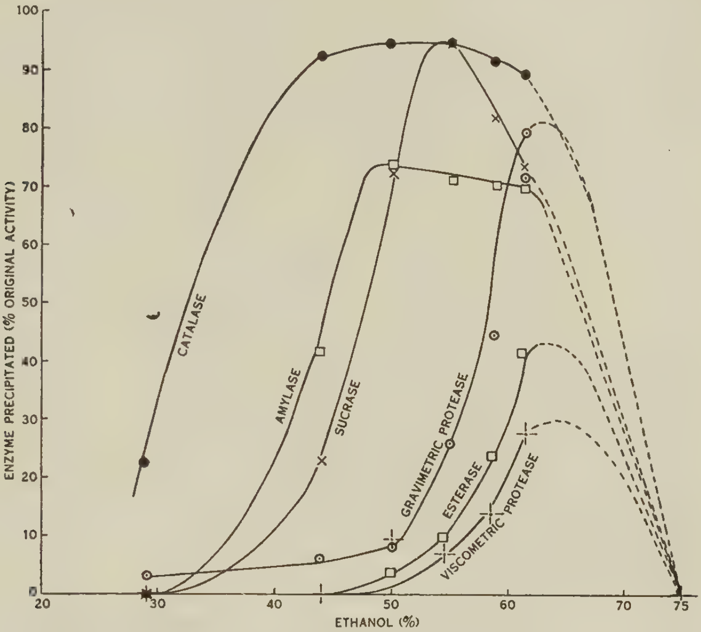

Fig. 6.—Ethanol precipitation curves in presence of 0.1M glycine at pH 4.0.

TABLE 1

EFFECT OF PRESENCE OF 0.1M AMINO ACIDS DURING PRECIPITATION OF MOULD ENZYMES WITH ETHANOL

The results are compared with those obtained in $0 \cdot 1$M acetate (Fig. 5)

Amino Acid	Sequence of Appearance of Enzymes
Glycine	As with acetate*
Lysine	Sucrase and amylase precipitation delayed†
δ-Aminovaleric acid ..	As with lysine†
Valine	As with acetate
Arginine	Amylase partly precipitated at low ethanol concentration. Sucrase precipitation delayed
Cysteine	As with acetate but all enzymes first precipitated at higher ethanol concentrations

*Enzymes become soluble at high ethanol concentrations.

†Two peaks of maximum precipitations of sucrase.

p-aminophenol was slightly less effective in repressing precipitation, though catalase was the only enzyme precipitated in any quantity.

Benzylamine, on the other hand, behaved in much the same way as methylamine (Fig. 8) and although piperidine repressed precipitation at low ethanol concentrations it allowed considerable precipitation at higher ethanol concentrations. The divergence in behaviour in the presence of piperidine from that observed in the presence of a typical aliphatic amine may be due to the

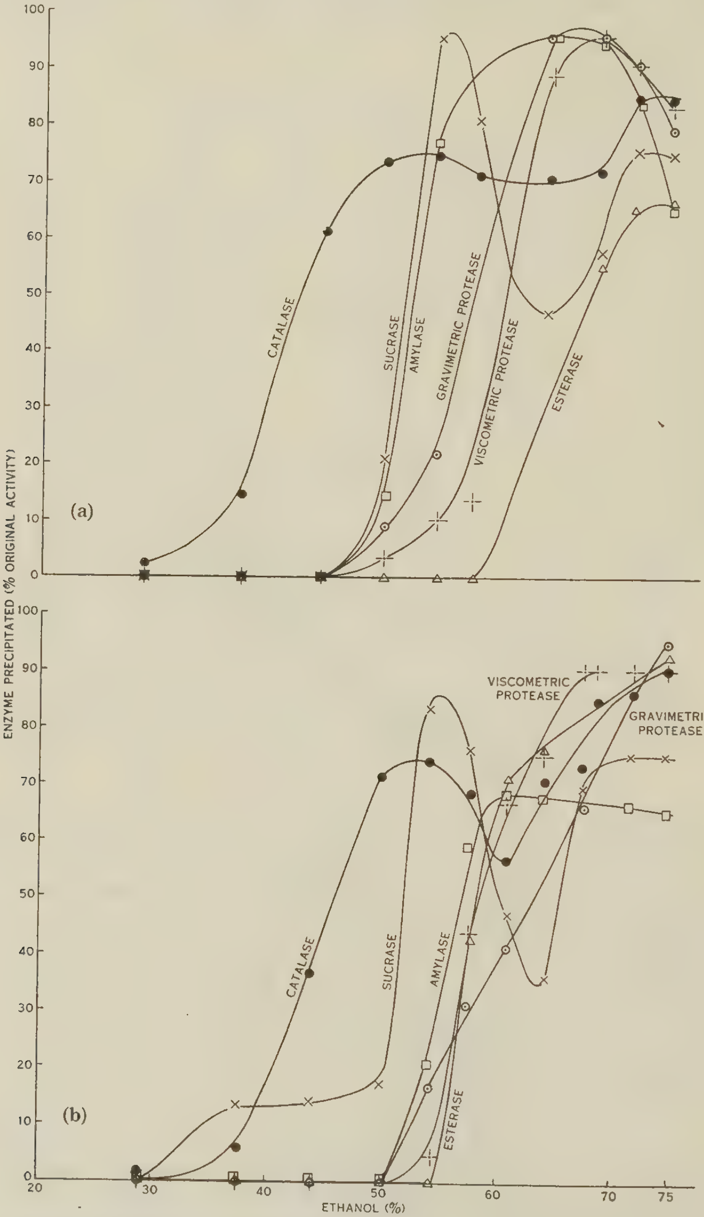

Fig. 7.—Ethanol precipitation curves in presence of 0.1M ammonium chloride (*a*) and methylamine hydrochloride (*b*) at pH 4.0.

presence of small amounts of pyridine. The most noteworthy effect of benzylamine was the suppression of precipitation of amylase, or alternatively its inactivation.

At low concentrations of the aromatic amines (aniline and pyridine) it is possible to obtain considerable amounts of precipitate, particularly at low ethanol concentrations. Different enzymes show differing tendencies to remain in solution at high ethanol concentrations under these conditions and it is possible to obtain partial separation of the enzymes in this way. The preparation of solutions containing the gravimetric protease in the absence of viscometric activity, using this method, has already been described (Crewther and Lennox 1953). However, a more general application of the method to the fractionation of culture filtrates has proved difficult owing to considerable variation between batches of filtrate. For this reason acetate buffer was used for the subsequent experiments.

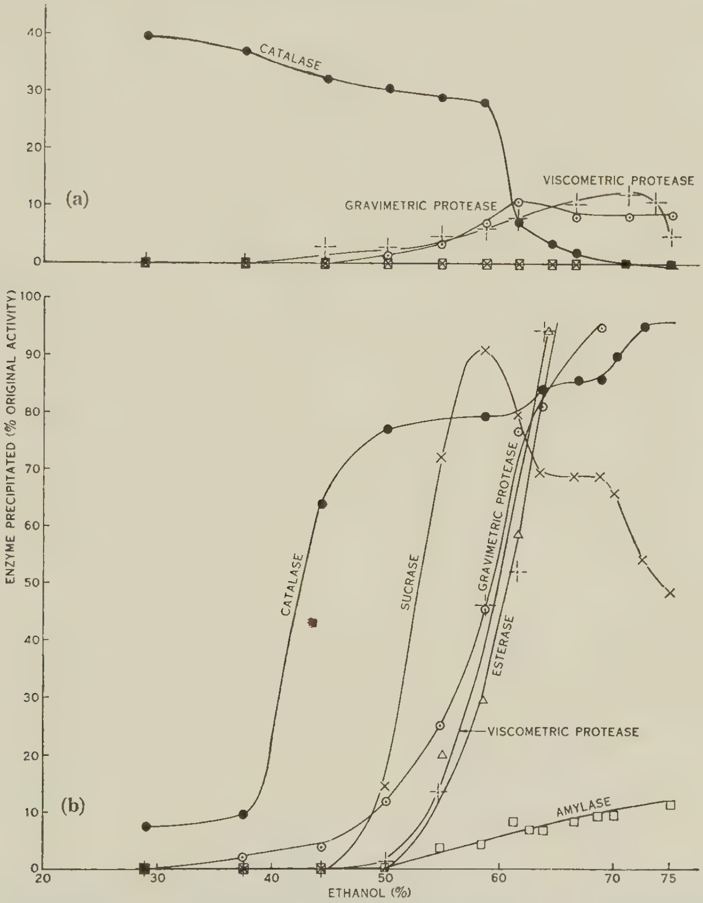

Fig. 8.—Ethanol precipitation curves in presence of 0.1M *p*-aminophenol (*a*) and benzylamine (*b*) at pH 4.0.

Low-temperature fractionation of an enzyme concentrate in the presence of 0.05M acetate, to give a pH of 4.7, yielded solutions having the activities shown in Table 2. Estimation of the total solids, protein N, and reducing substances (after acid hydrolysis) indicated the presence of carbohydrate in each fraction, amounting to approximately 15 per cent. of the total solids. Estimation of the total N indicated that the remaining 85 per cent. was almost entirely protein. Jermyn (1952) has identified the hexoses in a hydrolysate of the mould polysaccharide as mannose and galactose. The results in Table 2 indicate that catalase, the proteases, and possibly esterase are the major constituents of the protein in the concentrate.

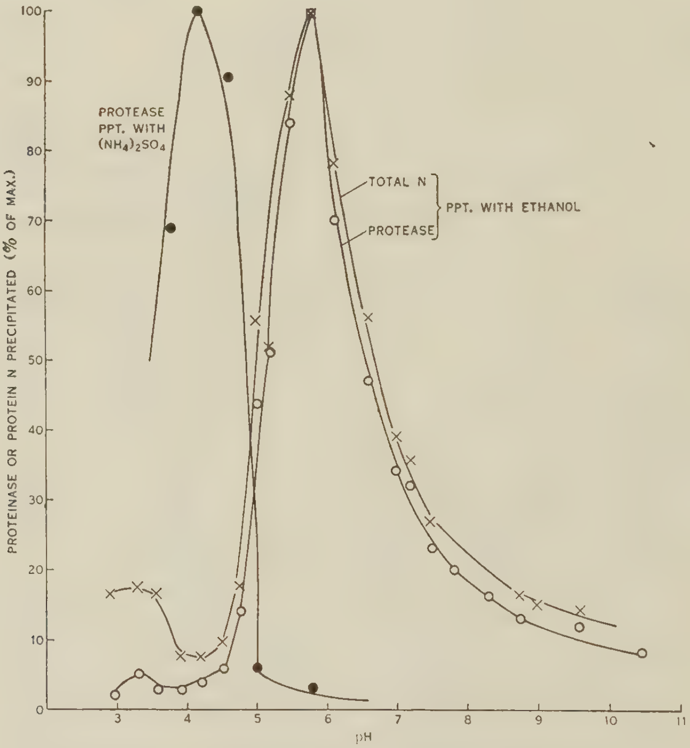

Fig. 9.—Effect of pH on viscometric protease and protein precipitation from a protease-rich fraction.

(*d*) *Subfractionation and pH-Solubility Relationships of Protease-rich Fractions*

A fraction obtained from the enzyme concentrate by precipitation with ethanol in the presence of acetate buffer at pH 4.0 over the range of ethanol concentrations 58-64 per cent., was re-precipitated with 2 vol. of ethanol, dissolved in water, and precipitated with 2.8M $(NH_4)_2SO_4$ at different pH values. Figure 9 shows that viscometric protease was precipitated maximally at a higher pH value than with crude enzyme solutions (Fig. 1) and the pH range was greatly restricted. When 67 per cent. ethanol was used as precipitant for a similar experiment (Fig. 9) the pH of maximum precipitation was again higher than that for crude enzyme (Fig. 4) and the pH range of precipitation was decreased.

Ethanol subfractionation of fractions 5 and 6, obtained by fractionation of mixed enzyme concentrate at pH 4.0, yielded the results shown in Figure 10.

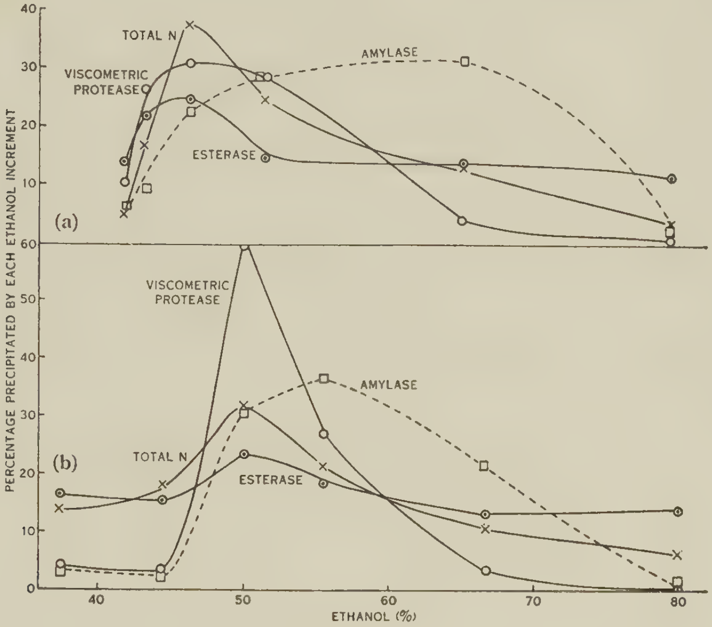

Fig. 10.—Subfractionation of protease-rich fractions with ethanol. (*a*) Subfractionation of fraction 5 (51-57 per cent. ethanol); (*b*) subfractionation of fraction 6 (57-61 per cent. ethanol).

TABLE 2

FRACTIONATION OF MOULD CULTURE CONCENTRATE WITH ETHANOL IN pH 4.7 ACETATE BUFFER

Enzyme activities expressed as percentage of total precipitable activity

Fraction (% ethanol v/v)	Catalase	Sucrase	Amylase	Phosphatase	Gravimetric Protease	Viscometric Protease	Esterase	Total N
0-50	97	32	18	9	2	Nil	4	19
50-56	3	40	22	23	18	2	4	8
56-64	Nil	25	49	53	32	22	17	23
64-69	Nil	3	9	9	35	41	46	33
69-74	Nil	Nil	2	6	13	35	29	17

The pH values of the fractions were not altered from their initial values of 4.6 and 4.8 respectively. Owing to the high concentration of viscometric protease in these fractions it precipitated at low ethanol concentrations, particularly in fraction 5.

Although maximum esterase activity was detected in the richest protease subfraction, almost as much was also detected in the later subfractions.

(c) *Formation of Protease-rich Crystals by Addition of Ammonium Sulphate to Ethanol Fractions*

Each of the fractions obtained by ethanol fractionation of the concentrate was dissolved in a minimum volume of water and treated with 4M ammonium sulphate until a faint cloud appeared. This was filtered off and the solutions were held at 2°C for several days. The fractions with the greatest protease content were then found to contain needle crystals either free or in rosettes, whereas those in which catalase or the carbohydrases preponderated produced granular material.

On suspending the crystalline precipitate in water, pronounced double refraction of flow was observed and some fractions, which appeared to be granular, also showed this phenomenon. In such instances the granules probably break up into minute needle crystals too small to be distinguished under the microscope.

TABLE 3

SUMMARY OF ACTIVITIES AND NITROGEN CONTENTS OF ENZYME SOLUTIONS AT VARIOUS STAGES IN A TYPICAL PURIFICATION PRIOR TO CRYSTALLIZATION FROM FRACTION 2

Solution	Volume (ml)	Viscometric Protease (arbitrary units/ml.)	Total Viscometric Protease (arbitrary units)	Non-dialysable N (mg/ml)	Viscometric Protease (arbitrary units/mg N)
Culture filtrate	20,000	25·0	500,000	0·21	119·0
Ethanol ppt. dissolved in water	500	268·5	134,250	2·50	107·2
After 1st dialysis (1 hr)	680	225·0	153,000	2·10	107·1
After 2nd dialysis (2 hr)	960	155·0	149,000	1·25	124·0
Fraction 1	200	131·0	26,200	1·35	97·1
Fraction 2	200	388·5	77,700	3·00	129·5

Further experiments showed that fractionation with ethanol into more than two fractions before crystallization was unnecessary and a better yield of crystals was obtained as follows: The concentrate from 20 l of culture filtrate (500 ml) was dialysed, mixed with 200 ml of 1.0M acetate buffer at pH 4.0, diluted to 2 l, and cooled to 2°C. An equal volume of ethanol* at −20 to −25°C was then added with agitation and the mixture held at about −10°C for 30 min. The precipitate was removed in an imperforate basket centrifuge in the cold room at −5°C and the supernatant precipitated with further ethanol (2 l). The second precipitate was recovered in the centrifuge and dissolved in a minimum volume of water (usually 200 ml) to yield a solution at pH 4.6 which, on addition of ammonium sulphate as described above, produced a heavy deposit of crystals. Yields of up to 10 g of crystalline material have been obtained from 20 l of culture filtrate. A summary of the volumes of solution, viscometric protease activities, and non-dialysable N contents up to the stage of crystallization is reported in Table 3.

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* The optimal ethanol concentrations for fractionation of different batches have been found to vary considerably. The best criterion for determining the limit of ethanol concentration for the first fraction is the appearance of a white precipitate in the rather brownish precipitate which is first formed.

The crystals have been found to possess many of the characteristics of other protein crystals produced by salt precipitation. They are formed from the enzyme solution slowly over periods of several weeks, and likewise they dissolve slowly on suspension in water. They have a much lower solubility than the amorphous material obtained by rapid precipitation with ethanol under similar conditions. Cooling the enzyme-ammonium sulphate solution is not necessary for the production of crystals, though there is an increase in the solubility of the crystals with increasing temperature.

Crystallization was found to take place over the pH range 4.0-5.2 with greatest yields of crystalline material and best crystal form at pH 4.6-4.7 (Fig. 11). In general, inadequate dialysis or crystallization under suboptimal conditions resulted in granules which sometimes had dark centres and developed into rosettes of crystals after prolonged standing at 2°C.

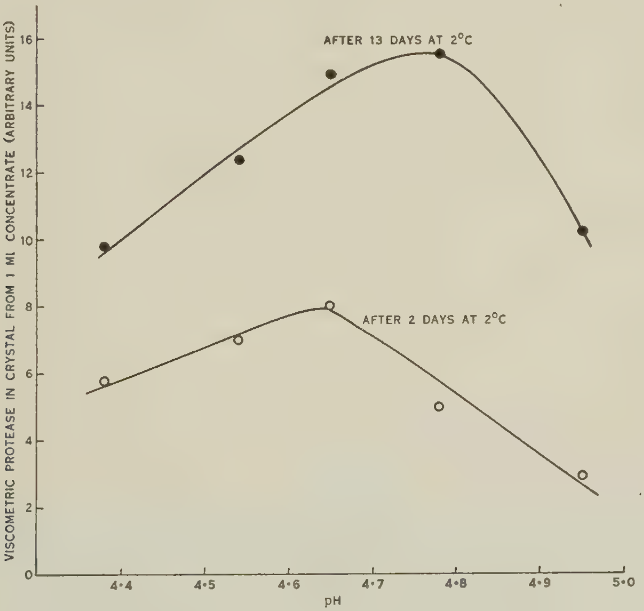

Fig. 11.—Activity of crystals obtained by precipitation with ammonium sulphate at various pH values.

By centrifuging 1-ml portions of the crystal suspension and diluting both the deposit and supernatant to 10 ml for measurement of enzyme activity, most of the viscometric protease in the fraction was shown to be precipitated in the crystals.

(*f*) *Composition of the Enzyme Crystals and Recrystallization*

Recrystallization was complicated by the presence of a considerable proportion of ammonium sulphate in the crystals. Addition of 4M ammonium sulphate at pH 4.7 to concentrated solutions of the crystals yielded non-crystalline precipitates. Dialysis of a solution of crystals and freeze-drying of the protein solution made possible the testing of different protein concentrations under the usual conditions of crystallization. However, although hexagonal plates were obtained from the more concentrated protein solutions, no needle forms appeared even after seeding with needle crystals.

Table 4

COMPOSITION OF CRYSTALS AND SUPERNATANT LIQUID DURING THE PROGRESS OF RECRYSTALLIZATION

Enzyme Preparation	Enzyme Activity (arbitrary units/mg total N)				Ammonia N (mg/mg total N)
	Viscometric Protease	Gravimetric Protease	Esterase	Glycylglycine Dipeptidase	
1st Crop crystals	15·1	5310	0·34	0·157	0·69
1st Supernatant	12·9	5770	0·26	0·178	0·72
Ratio of activity:					
$\frac{\text{1st Supernatant}}{\text{1st Crystals}}$	0·86	1·09	0·77	1·13	(1·04)
2nd Crop crystals	13·1	3470	0·53	0·124	0·71
2nd Supernatant	18·0	3140	0·57	0·089	0·77
Ratio of activity:					
$\frac{\text{2nd Supernatant}}{\text{2nd Crystals}}$	1·37	0·91	1·08	0·72	(1·08)
3rd Crop crystals	11·3	1740	0·51	0·057	0·78
3rd Supernatant	12·0	1340	0·43	0·028	0·80
Ratio of activity:					
$\frac{\text{3rd Supernatant}}{\text{3rd Crystals}}$	1·06	0·77	0·84	0·50	(1·03)

A satisfactory yield of recrystallized material in the form of needle crystals was finally accomplished by dissolving enzyme crystals in a minimum volume of distilled water at 30°C (about 0.2 g/ml) leaving a few crystals undissolved. On cooling to 2°C and holding at that temperature for a few days, a heavy precipitate formed consisting of large rosettes of long needle crystals; crystallization continued for several weeks. By holding the solution for 18 hr at 30°C prior to cooling, the traces of amylase and sucrase, which are always present after the first crystallization, were almost completely eliminated.

The crystalline preparations have a number of enzymic activities of which the gravimetric and viscometric proteases, esterase, and glycylglycine dipeptidase appear to be the most important. During the recrystallization process it is possible to recover the crystals at intervals as they are formed and examine their composition. The data for Table 4 were obtained in this way. The crystalline preparation used for this particular recrystallization contained a much higher proportion of the gravimetric protease in relation to the esterase and the viscometric protease than most others. On recrystallization the first crystals formed had more viscometric protease and esterase activity per mg N than the supernatant, but less gravimetric protease and dipeptidase activity. In the second and third crops of crystals, however, the trend was reversed.

A similar experiment was conducted with a crystal preparation having a ratio of activity of the gravimetric to the viscometric protease of 110 : 1 as compared with the ratio of 450 : 1 in the preparation to which Table 4 refers. In this instance the ratio of activities of the gravimetric to the viscometric proteases increased during recrystallization to 135. Crystals prepared under suboptimum conditions contained a low percentage of protein. As shown in Table 5, with repeated recrystallization the enzymes were enriched at the expense of the ammonia N. This approached a ratio to the total N of 0.7, the figure attained also in the other recrystallizations.

Table 5

REPEATED CRYSTALLIZATION OF A MIXED CRYSTAL PREPARATION

Enzyme Preparation	Enzyme Activity (arbitrary units/mg total N)			Ammonia N (mg/mg total N)
	Viscometric Protease	Gravimetric Protease	Esterase	
Original crystals	11·5	700	0·32	0·86
Once recrystallized	12·2	770	0·33	0·84
Twice recrystallized	34·2	4770	0·82	0·70

Recrystallizations have been successfully carried out on crystal preparations after holding in the dry state for 2 years. Both protease activities and the esterase activity of the crystals are unchanged at room temperature but the glycylglycine dipeptidase loses activity and finally disappears.

Solubility experiments were complicated by the presence of ammonium sulphate in the crystals. Varying amounts of a crystal preparation were shaken in a bath for periods of 1.5 and 3 hr at 18°C with 2.5M ammonium sulphate, which had previously been found to cause partial solution of the crystals. The relationship between the viscometric protease activities of the supernatant solutions and the amount of crystalline material used is shown in Figure 12. The appearance of a peak of maximum solubility indicates the effect of ammonium sulphate dissolved from the crystals on the amount of protein remaining in solution.

IV. Discussion

The pH-solubility curves of various proteins in ethanol-water mixtures and in concentrated salt solutions have been shown to have sharp solubility minima (Green 1931; Surgenor *et al.* 1949), and the method devised by Distèche (1948) for obtaining crystalline preparations from mixtures of proteins depends for its success on each component having low solubility over a rather narrow band of pH values. Thus the pH-solubility curves obtained with dialysed mould enzyme concentrate (Figs. 1-4) were atypical, whereas on repeated fractionation and precipitation with ethanol typical narrow pH ranges of low solubility were obtained (Figs. 9 and 10). These results suggest that the dialysed concentrate contains a component or components which interfere with the solubility characteristics of the proteins in ethanol and salt solutions, this component being removed during fractionation of the proteins with ethanol. Recent work by Gillespie and Woods (1953) has demonstrated that interference with ethanol fractionation by the considerable amount of non-dialysable ionic material in the dialysed concentrate is responsible for these results.

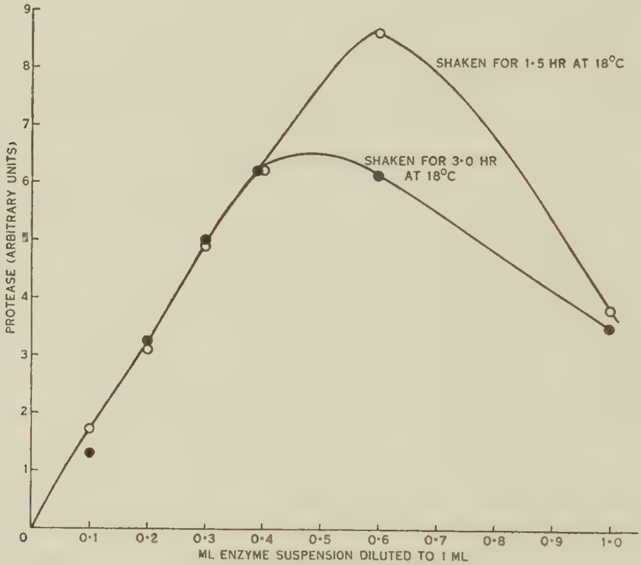

Fig. 12.—Solubility of viscometric protease in 2.5M ammonium sulphate.

Some difficulty arises in interpreting the different effects of aromatic and aliphatic amines. The most reasonable explanation for the increase in solubility of the proteins in ethanol-water mixtures in the presence of aniline, *p*-aminophenol, *p*-phenylenediamine, or pyridine appears to be combination of protein with the compound through its amino group, either by hydrogen bond formation or by ionic attraction, with a consequent masking of the polar groups of the protein. If the effect were produced by the ionic form of the compound, no great difference in ethanol precipitation of the enzymes would be expected in the presence of either aromatic or aliphatic amines since both types are largely ionized under the experimental conditions. Phenyl ammonium ions and benzyl ammonium ions should then have analogous effects.

It is pointed out by Pauling (1945) that an ionized amino group will donate a proton to form a hydrogen bond more readily than an un-ionized amino group. For this reason the un-ionized form of aniline, which resonates with forms having a positive charge on the nitrogen and a negative charge on the aromatic ring, would form hydrogen bonds more readily than an un-ionized aliphatic amine in which the nitrogen is practically uncharged. The overall positive charge of the ionized form of the amines may prevent complex formation with the proteins, most of which are positively charged under the experimental conditions. The smaller solubilizing effects of substituted anilines can be explained in terms of the greater polarity of the aromatic ring in these compounds. The solubilizing effect of glycine on proteins in ethanol-water mixtures has been observed previously and employed in protein fractionation (Cohn *et al.* 1950).

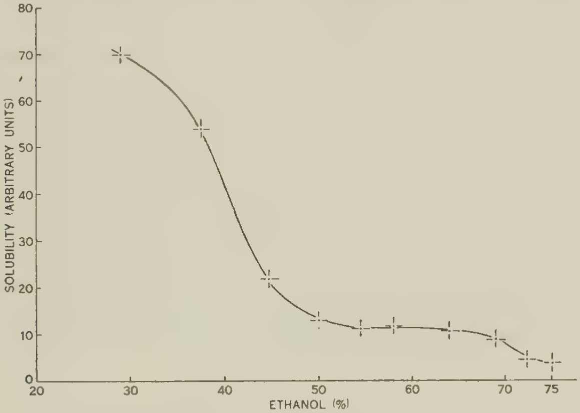

Fig. 13.—Ethanol solubility of catalase in presence of of 0.1M ammonium chloride.

The fractionation results have provided further evidence for the view already proposed (Crewther and Lennox 1953) that the gravimetric and viscometric proteases are separate enzymes. There is a marked tendency for the gravimetric protease to commence precipitation at a lower ethanol concentration than the viscometric protease. The distribution of the two enzymes in crystalline preparations and supernatant solutions is also markedly different.

There is also evidence that catalase consists of two enzymes, as shown by the typical two-component solubility curve obtained by converting the precipitation curve for catalase, in the presence of ammonium chloride, to a curve relating the solubility of catalase in the ethanol-water mixtures to the ethanol content (Fig. 13). The dual peaks obtained when precipitating sucrase in the presence of aliphatic amines do not correspond with the curves expected for a two-component system and although more than one enzyme is involved (Gillespie, Jermyn, and Woods 1952) it seems probable that one component redissolved at high ethanol concentrations.

Gillespie, Jermyn, and Woods (1952) have shown that the difficulties encountered in obtaining relatively pure preparations of the mould proteases are attributable to the complexity of the various enzyme systems present in the culture filtrates. However, paper electrophoresis has shown that fractionation under the conditions described in this paper for preparing crystalline material does, in fact, separate a number of major components of the system (Gillespie and Woods 1953).

On recrystallization from water the composition of the crystals is found to be similar in most enzyme activities and in ammonium ion to that of the solution remaining (Table 4), though as successive batches of crystals are removed from a concentrated liquor there is found to be a decrease in the activity of the gravimetric protease and the glycylglycine dipeptidase per mg total N which probably indicates a progressive inactivation and breakdown of these enzymes. The slight increase in the ammonia N content per mg total N may be due to protein decomposition. In general, however, the constancy of the ammonia content of the crystals is remarkable and there seems little doubt that ammonium sulphate forms part of the crystal structure. It seems probable that the enzymes form a series of mixed cystals having lower solubilities than the component proteins or ammonium salts and that several eutectic mixtures are possible. It is not unlikely that many well-known salt-precipitated enzyme crystals are also mixed crystals of the salts and proteins.

Astrup and Alkjaersig (1952) have recently published a classification of proteolytic enzymes in terms of their inhibition by various specific inhibitors and surface active agents. They showed that the crystalline proteases of *A. oryzae*, described in the present paper, were atypical in that they were activated by both cationic and anionic detergents. The presence of at least two, and possibly three, proteases in the crystalline material may explain this apparent anomaly.

V. Acknowledgments

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ENZYMES OF *ASPERGILLUS ORYZAE*

V. ETHANOL FRACTIONATION AT LOW IONIC STRENGTHS*

By J. M. Gillespie† and E. F. Woods†

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Summary

Moving boundary and paper electrophoresis were applied to crude and deionized enzyme preparations from cultures of *Aspergillus oryzae.* Five main protein components were observed by both methods and four additional minor components were demonstrated with the paper method.

After paper electrophoresis the main protein component was found to correspond with a protease and an esterase with isoelectric points at pH 5.1. A second protease less active than the other protease in digesting gelatin, with an isoelectric point of 4.2, and a second esterase of isoelectric point 3.9, were also characterized. Sucrase appeared as a single peak whereas amylase and cellulase showed at least six and seven peaks respectively.

Solubility curves of dialysed crude mould enzyme in ethanol-water mixtures at low temperatures were atypical in that variation of pH and ionic strength had little effect on the solubility owing to the presence of 70 per cent. ionic material. Removal of this material with ion exchange resins also removed about 50 per cent. of the enzyme protein, whereas electrodialysis removed it almost completely without loss in enzyme activity. Material treated in this way was found to provide typical pH-solubility curves having well-defined solubility minima near the isoelectric point of the major protease (pH 5.1). Using this data two proteases were separated from each other and, after reprecipitation at the isoelectric point, the major protease was obtained electrophoretically pure.

A crystalline preparation of mould protease was shown to contain a single component representing approximately 90 per cent. of the protein present and having mobility corresponding to that of the major protease.

I. Introduction

Cohn *et al.* (1946, 1950) have developed the basic procedures for separating plasma proteins by ethanol fractionation at low temperatures and low ionic strengths and their methods have been used for enzyme fractionation by Ellis and Fruton (1951) and Askonas (1951) and in the investigations described in the present paper.

By ethanol fractionation followed by salting-out Crewther and Lennox (1950) prepared crystals containing protease from culture filtrates of a strain of *Aspergillus oryzae.* In subsequent papers Maxwell (1952*a*, 1952*b*) described the growth conditions for maximum enzyme production by this strain, and

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Crewther and Lennox (1953*a*, 1953*b*) published further information concerning the nature of the enzymes formed and the methods employed in the early fractionation experiments. The techniques of filter paper electrophoresis and chromatography (Gillespie, Jermyn, and Woods 1952; Jermyn 1953) have shown the extreme complexity of the culture filtrates. They contain not only many different types of enzymes, but also several components specific for a single substrate; for example, there are at least six amylase components. Fractionation was further complicated by the presence of salts in the mould culture filtrate even after dialysis. Further purification was therefore a necessary preliminary to successful fractionation.

The present paper reports the results of further fractionation studies of the enzymes produced by *A. oryzae*. As in earlier studies, ethanol was used as the precipitant but additional means were used to remove ionic material, thereby enabling solutions of known ionic strength to be fractionated. The homogeneity of the fractions has been examined by electrophoresis.

II. METHODS

(*a*) *Enzyme Estimations*

Most of the protease estimations were made by the azoprotein technique of Charney and Tomarelli (1947) using sulphanilamide azocasein as substrate at pH 8. To cover a wider pH range the sulphanilic acid azo-albumin substrate of Tomarelli, Charney, and Harding (1949) was also employed. The method of Anson (1938), using haemoglobin and casein as substrates, the gravimetric gelatin technique of Crewther (1952), and the viscometric gelatin method of Lennox and Ellis (1943) were used also to provide comparative data with other substrates. The estimation of esterase, amylase, and sucrase is described by Crewther and Lennox (1953) and cellulase (C_x) by Jermyn (1952).

(*b*) *Protein Estimation*

The modified biuret procedure of Mehl (1945) was used initially in which each series of readings was standardized against crystalline bovine plasma albumin. For most of the estimations, however, a procedure based on measurement of the optical density at 278 mμ was employed. Electrodialysed mould enzyme preparations had an $E_{1\,\mathrm{cm}}^{1\%}$ at 278 mμ of 8.6, whereas crude mould enzyme had an extinction coefficient of nearly 14. There was remarkably little variation in extinction coefficient between mould enzyme fractions.

(*c*) *Electrophoresis*

(i) *Moving Boundary Electrophoresis*.—The crude enzyme and electrodialysed preparations in 2 per cent. solution were dialysed for 48 hr at 1° C against 0.1 $\Gamma/2$ veronal buffer at pH 8.6. Electrophoresis was carried out at this pH in a Tiselius apparatus[*] at 1°C for 3 hr with a current of 15 mA.

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[*] LKB Type 3021 from LKB-Produkter, Stockholm 12, Sweden.

(ii) *Filter Paper Electrophoresis.*—A modification was used of the method of Cremer and Tiselius (1950) as described by Woods and Gillespie (1953). The distribution of protein along the paper was determined by the dyeing procedure of Durrum (1950) and also by the less sensitive spectrophotometric measurement of water eluates at 278 mμ. Owing to differences in affinity of different proteins for dye and differences in the absorption of radiation at 278 mμ, neither method provides an absolute measure of protein distribution.

The distribution of enzymes was followed by cutting the paper transversely into 1-cm strips, eluting with water, and estimating the activity of the eluates. Many enzymes were extraordinarily labile in the dried state on the paper after electrophoresis, the viscometric protease being inactivated during electrophoresis (Crewther and Lennox 1953*a*), inactivation of catalase and alkaline phosphatase occurring within 1 hr at room temperature and many of the other enzymes being inactivated within 18 hr after drying. The strips were therefore stored at −20°C until required, to retain full activity of the enzymes studied.

(*d*) *Dialysis and Electrodialysis*

Dialysis in cellophane tubing against distilled water was ineffective in removing all the salts from crude mould enzyme. Electrodialysis, on the other hand, was successfully used for this purpose. A 10 per cent. solution was electrodialysed between concentric cellophane bags against running distilled water for 8-12 hr at 400 V with an initial current of 5 mA and a final current of less than 0.5 mA. Least enzyme damage occurred at temperatures approaching 0°C. After electrodialysis a small quantity of globulin-like non-enzymic protein was centrifuged off and the supernatant freeze-dried. The yield corresponded to 30 per cent. of the material in solution before electrodialysis.

(*e*) *Ion Exchange*

A column of Amberlite MB1 monobed ion exchange resin* was used. This is composed of equal parts by volume of Amberlite IR-120 and IRA-400 separately regenerated and then mixed. The progress of deionization was followed by measuring the conductivity of the issuing protein solution at 1000 c/s. The monobed resin was regenerated by floating apart the two resins with an inorganic salt solution of suitable specific gravity and then washing and treating with acid or alkali.

(*f*) *Freeze-drying*

All precipitates and solutions containing protein were dried as quickly as possible to remove the solvents, minimize digestion by the protease components, and convert the protein to a stable form suitable for storage. The drying apparatus used was a modification of that designed by Flosdorf and Mudd (1935). The dry protein was stored at −5°C.

(*g*) *Solubility Studies*

Solubility studies were made by adjusting aliquots of an aqueous solution to the desired pH, temperature, ionic strength, ethanol concentration, and protein concentration. The resulting suspensions were allowed to equilibrate for 24 hr at the desired temperature, after which the solid phase was separated by centrifugation at the same temperature and the concentration of protein in solution estimated. The solid phase was in the amorphous state. The ethanol concentrations are recorded as percentages of the commercial (95 per cent.) solvent in the system.

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* Röhm and Haas Co., Washington Square, Philadelphia 5, Pa., U.S.A.

(*h*) *pH Measurement*

The pH was controlled wherever possible by the use of buffers to yield the desired ionic strength. The recorded pH values do not refer to the ethanol-water mixtures at low temperatures but to solutions obtained by dilution of these systems to ethanol concentrations of 5 per cent. or less at room temperature; 0.02M NaCl was used as the diluent in systems of low ionic strength.

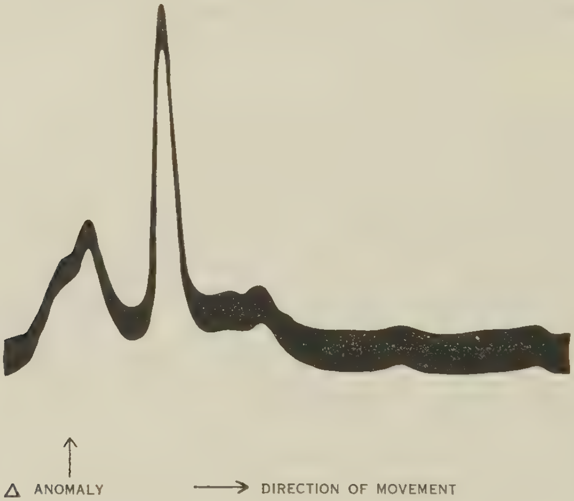

Fig. 1.—Moving boundary electrophoresis of electrodialysed mould enzyme in veronal buffer $\Gamma/2$ 0.1, pH 8.6, 2 per cent. protein, 180 min, 15 mA, ascending boundary.

(*i*) *Preparation of Mould Culture Filtrate*

The mould culture filtrate was prepared, as described by Maxwell (1952*a*, 1952*b*), by growing a selected strain of *A. oryzae* for 10 days at 22°C. It was a clear amber fluid having a pH of approximately 7, containing about 0.1 per cent. protein, and being approximately 0.5M with respect to salts.

(*j*) *Preparation of Dry Crude Mould Enzyme*

Pooled culture filtrate (20 l) was dialysed against running tap water for 2 days at 10°C, whereby the salt concentration was reduced to a constant value, then cooled to 0°C, and maintained at that temperature until it was converted to an ice slurry. A coil through which ethanol passed at – 25°C was immersed in the solution to facilitate cooling. Ethanol at – 20°C was then run in beneath the surface from two jets at a rate of 200 ml per min with vigorous stirring to give a final concentration of 60 per cent. As the ethanol was added the temperature gradually fell to −20°C, at which it was maintained for 8 hr. The precipitate was collected in a refrigerated Sharples super-centrifuge at −20°C, suspended in an ice-water slurry and freeze-dried to yield 20-30 g of light powder which was sealed in bottles and stored at −5°C. There was no loss in enzyme activity during a period of 12 months.

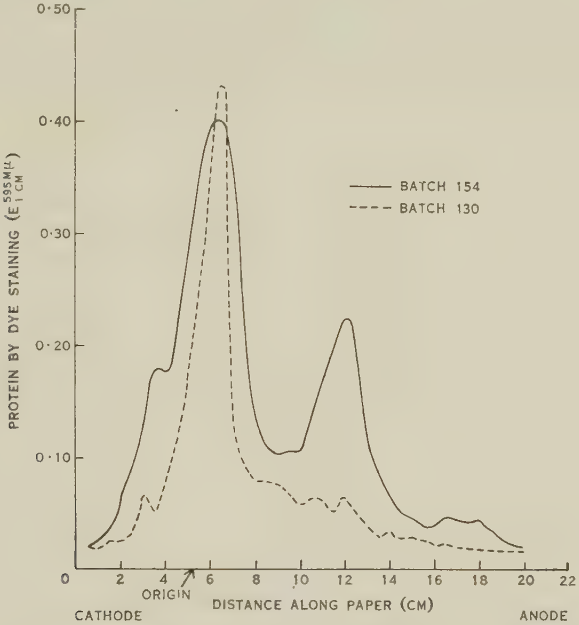

Fig. 2.—Comparison of the protein distribution by paper electrophoresis of two batches of electrodialysed mould enzyme in veronal buffer, Γ/2 0.025, pH 8.6 for 6 hr at 7.15 V/cm.

III. Results

(*a*) *Moving Boundary Electrophoresis*

Figure 1 shows the electrophoretic pattern of electrodialysed mould enzyme in veronal buffer Γ/2 0.1, pH 8.6, 2.0 per cent. protein. Five components are visible and a similar though less distinct pattern was obtained before electrodialysis. The electrophoretic mobility of the major component was found to correspond with that of the crystalline material of Crewther and Lennox (1950). The electrophoresis pattern gives no indication of the great complexity of the mould culture filtrates in terms of the enzyme components reported by Gillespie, Jermyn, and Woods (1952) and in the previous papers of this series.

(*b*) *Paper Electrophoresis*

Nine components can be seen in typical paper electrophoresis experiments but these are not always obvious in graphs plotted from absorption measurements on eluates.

(i) *Variability Amongst Batches.*—Mould enzyme was produced in weekly batches and although growth conditions were apparently identical, considerable variation occurred from one experiment to another. Such variations could be detected by enzyme estimation and also by paper electrophoresis. Electrophoretograms from two batches of enzyme, both run in veronal buffer pH 8.6 Γ/2 0.025, are shown in Figure 2. It can be seen that one of the main protein components is absent from batch 130. Some of the minor components are also missing or occur at a lower concentration. Similar differences were also reflected in the enzymic composition, one batch, for example, being rich in protease and poor in amylase whilst the reverse was true for another batch.

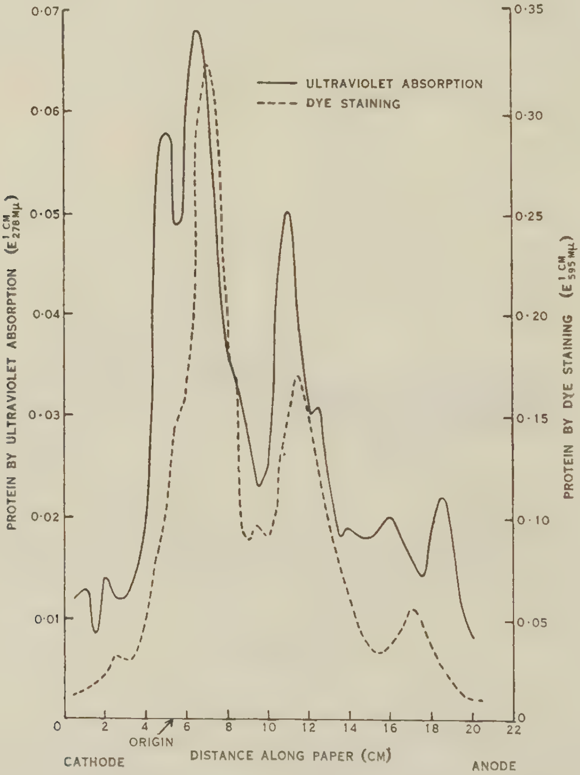

Fig. 3.—Protein distribution in electrophoretograms of electrodialysed mould enzyme, determined by two methods. Run in borate buffer Γ/2 0.1, pH 8.6 for 6 hr at 7.15 V/cm.

(ii) *Protein Distribution Along the Paper After Electrodialysis.*—Electrodialysed enzyme was electrophoresed on duplicate strips of paper at pH 8.6 in Γ/2 0.1 borate buffer at 7.15 V/cm for 360 min and the protein distribution along the paper was measured by the two methods. It can be seen from Figure 3 that the main protein components, as measured by each method, have migrated to almost identical positions on the paper but the relative heights of the peaks vary. A more distorted pattern was obtained with the mould enzyme before electrodialysis.

(iii) *Distribution of Enzymes Along the Paper*.—As reported earlier, the mould culture filtrate contains multi-component enzymes (Gillespie, Jermyn, and Woods 1952). Before proceeding with further fractionation experiments, paper electrophoresis was employed to determine the complexity of the protease, esterase, sucrase, amylase, and cellulase and to ascertain the electrophoretic mobility of each component. Typical results of experiments on solutions of the dried electrodialysed mould enzyme are shown in Figure 4.

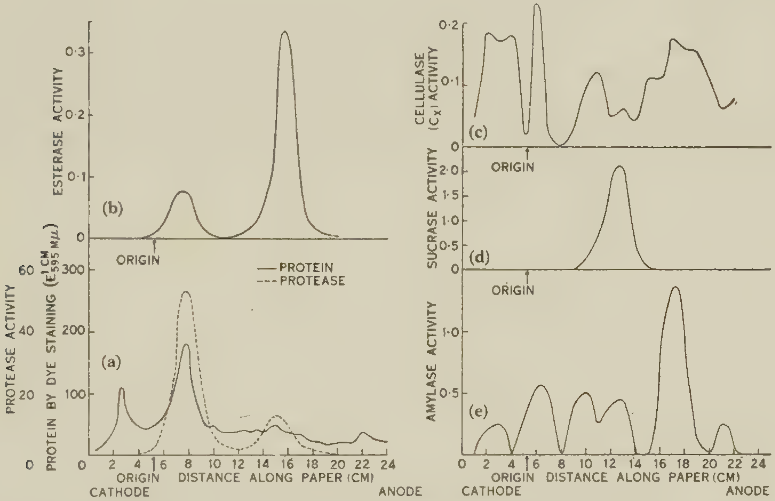

Fig. 4.—Enzyme distribution on paper electrophoretograms of electrodialysed mould enzyme. Run in veronal buffer Γ/2 0.025, pH 8.6 for 6 hr at 7.4 V/cm. Enzyme activities in arbitrary units.

Protease.—Using azocasein as a substrate the protease system was resolved into a large anodic component 2.5 cm from the origin and a smaller anodic component 8 cm from the origin. This experiment was repeated at pH values between 3 and 10 but no further resolution could be achieved. The results obtained at pH 8.6 and pH 5.0, using azocasein as substrate at pH 8.5 and 37°C, resemble those obtained using Anson's method with casein as substrate. Both methods gave similar twin peaks. The minor protease could be well distinguished from the major component for it gave barely measurable hydrolysis of gelatin, haemoglobin, and serum and egg albumins, whereas the latter readily attacked these substrates. The best separation of proteases was obtained at pH 5 in acetate buffer, one protease migrating to the anode, the other to the cathode. Their approximate isoelectric points are 4.2 and 5.1 (Woods and Gillespie 1953). Crewther and Lennox (1953*a*, 1953*b*) have shown that a third protease in the crude enzyme, which is capable of rapidly reducing the viscosity of gelatin, is inactivated during paper electrophoresis.

Esterase.—The mould enzyme esterase, which hydrolyses simple aliphatic esters, has been resolved into two components. The first, of isoelectric point pH 5.1, is closely associated with the major protease at a point 2.5-3.0 cm on the anodic side of the origin, the second is about 10.5 cm on the anodic side of the origin, has an isoelectric point of pH 3.9, and is not associated with protease activity. Between pH 4 and 10 the slow esterase component and the major protease component appear to travel together; they have not been separated by any method of fractionation yet employed.

Carbohydrases.—Whereas sucrase appeared as a single component, amylase was resolved into six well-defined components on paper electrophoresis. In the course of fractionation experiments evidence was obtained for the existence of nine amylases, all having different mobilities. From comparison of these results with those in Figure 4 it appears that some amylases with different solubilities in ethanol-water mixtures have approximately the same electrophoretic mobilities. Some of the amylase components were absent from some batches of mould enzyme having low total amylase activity. The multi-component nature of the cellulase is also evident from Figure 4.

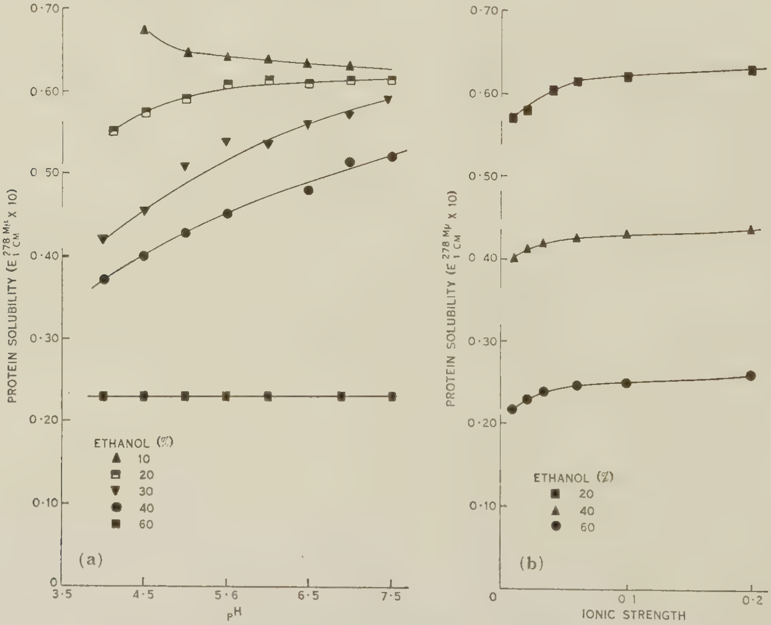

Fig. 5.—Solubility of crude mould enzyme at −5°C.

(*a*) In 0.01 Γ/2 salt solutions as a function of ethanol concentration and pH.

(*b*) At pH 4.5 as a function of ionic strength (sodium acetate) and ethanol concentration.

(*c*) *Fractionation Experiments*

(i) *Solubility Studies on the Mould Enzyme.*—The solubility of the mould enzyme preparation before treatment with ion-exchange resin or electrodialysis has been studied, using ethanol-water mixtures at −5°C under controlled conditions of pH and ionic strength with the solid phase in the form of amorphous protein. The results are presented in Figure 5. Variation in pH and ionic strength had very little effect on the solubility and no points of minimum solubility were observed. Subsequently it was found that the preparation contained only about 30 per cent. by weight of protein. The remainder was shown by Dr. M. A. Jermyn, of this laboratory, to comprise mainly salts of tartaric and valeric acids, bound more or less firmly to the protein. The ionic strength of the solution was therefore sufficient to mask solubility changes due to added electrolyte.

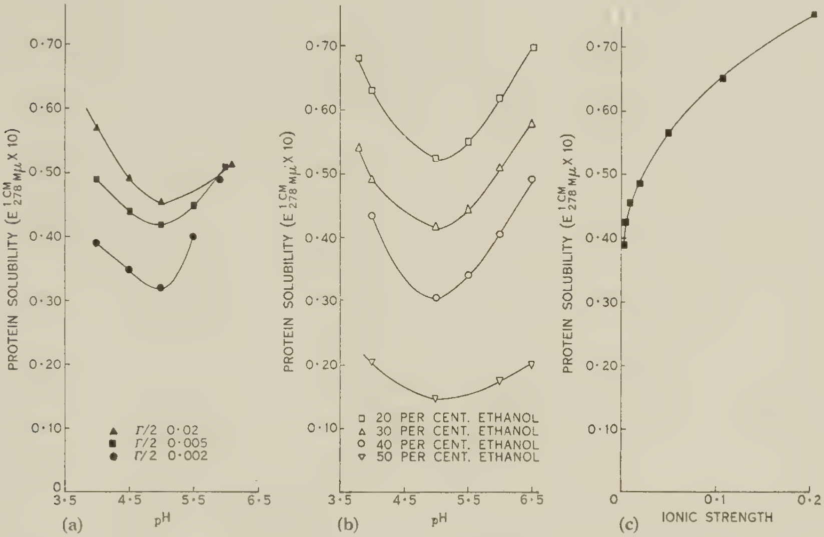

Fig. 6.—Solubility of electrodialysed mould enzyme at −5°C.
(*a*) In 30 per cent. ethanol as a function of pH and ionic strength.
(*b*) In 0.005 Γ/2 salt solutions as a function of pH and ethanol concentration.
(*c*) In 30 per cent. ethanol at pH 5 as a function of ionic strength of sodium acetate.

Following the recommendation of Edsall and Surgenor (personal communication 1950) a 10 per cent. aqueous solution of the mould enzyme was run through a column of mixed ion-exchange resin. Aliquots of the effluent were collected and the conductivity, protein concentration, and the protease, esterase, amylase, and sucrase activities were measured. The conductivity fell to approximately 1 per cent. of its initial value. When the enzyme activities of each aliquot of effluent were plotted graphically against the fraction number the peaks in the curves for some of the enzymes suggested that chromatographic separation may have occurred. Approximately 50 per cent. of the protein was lost during the operation, presumably by uptake on the resins, and this included certain enzymes, notably the viscometric protease, catalase, alkaline phosphatase, and some components of the amylase and β-glucosidase complexes. Furthermore, removal of non-protein material was not complete for the pooled effluent after freeze-drying still contained only 75 per cent. protein.

The solubility of some samples obtained in this way was studied in ethanol-water mixtures at −5°C under controlled conditions of pH and ionic strength. Though the protein became more soluble with increase in ionic strength, pH had little effect on the solubility. Because of the imperfect removal of small molecules and the loss of some of the enzymes this method was discontinued.

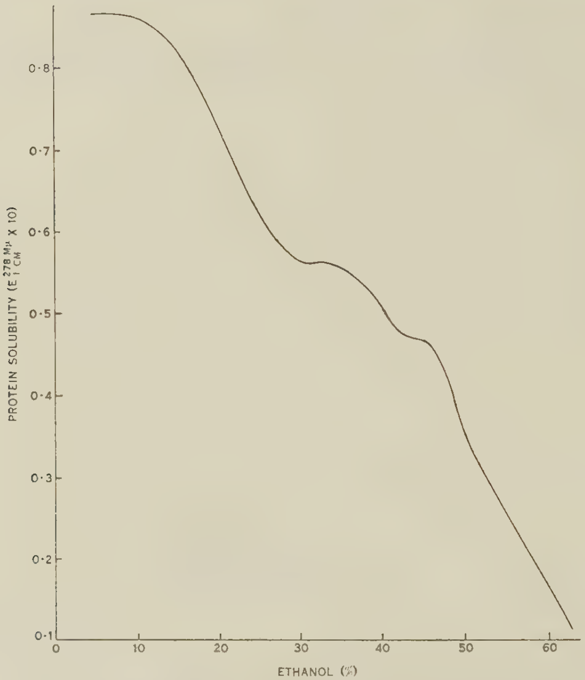

Fig. 7.—Solubility of electrodialysed mould enzyme at −5°C in acetate buffer pH 4.19, Γ/2 0.005 as a function of ethanol concentration.

The most successful method of deionizing the mould enzyme proteins was by electrodialysis. A 10 per cent. aqueous solution of the crude enzyme was electrodialysed, centrifuged to remove globulins, and freeze-dried. The yield of dry material corresponded to 30 per cent. of the starting product and it contained over 90 per cent. protein, according to the biuret method of protein estimation using albumin as a standard. It was a white powder soluble in water at room temperature to the extent of about 40 per cent. Most of the original enzyme activity was retained, the activity per unit weight in general increasing in proportion to the removal of inactive material during dialysis. This material apparently includes a compound having a high absorption coefficient at 278 mμ since the absorption fell from 14 to 8 during the treatment. On storage at 0°C for several weeks, certain enzymes, including catalase and alkaline phosphatase, commenced to lose activity and eventually became completely inactive. Previous storage experiments with the mixed enzymes in solution revealed similar loss in activity for these two components (Crewther and Lennox 1953*a*).

The solubility of electrodialysed enzyme in ethanol-water mixtures at −5°C under controlled conditions of pH and ionic strength is shown in Figure 6. The minimum solubility in acetate buffers is seen to occur near to pH 5.1, which is the isoelectric point of the major protease component and protein. The solubility in acetate buffers was markedly affected by changes in pH and ionic strength. Increase in ionic strength from 0.002 to 0.02 produced approximately 45 per cent. increase in solubility at pH 5.1 in 30 per cent. ethanol, whereas at pH 6.2 the salting-in effect was negligible. It was further noted that in the high ethanol concentration range, 50-60 per cent., pH and ionic strength had very little effect on solubility. The solubility curves are of the type often found with proteins (Koechlin 1952). Sodium chloride had virtually the same salting-in effect as sodium acetate but both effects were much less than those reported from similar experiments with several blood plasma proteins.

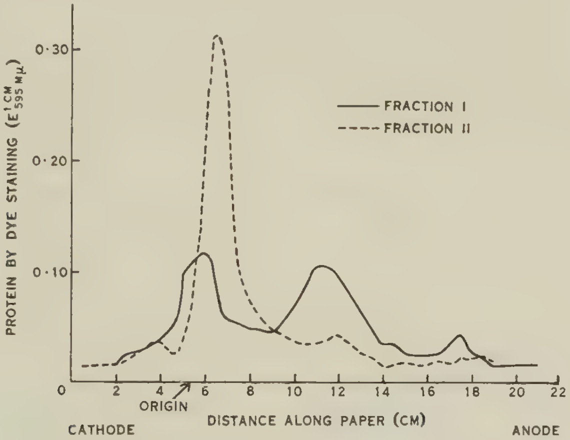

Fig. 8.—Paper electrophoresis of fractions I and II from electrodialysed mould enzyme in veronal buffer $\Gamma/2$ 0.025, pH 8.6, for 6 hr at 7.4 V/cm.

(ii) *Fractionation of Electrodialysed Enzyme*.—It is generally accepted that for satisfactory separation between proteins using ethanol fractionation a pH outside the range of isoelectric points is desirable. This was difficult to achieve in the present study, for above pH 7.0, the isoelectric point of one of the amylases, the proteins were too soluble, and below pH 3.9, the isoelectric point of the acid esterase, the enzymes were rapidly inactivated. A pH of 4.2 was therefore chosen as a compromise.

The solubility of the electrodialysed mould enzyme at this pH was measured in acetate buffer of ionic strength 0.005 at −5°C with 1 per cent. protein present over the range 0-60 per cent. ethanol (Fig. 7). Practically no decrease in solubility occurred until a level of 11 per cent. ethanol was reached, thereafter small increments in ethanol concentration caused a sharp decrease in solubility. Marked inflexions in the curve were obtained at ethanol concentrations of 35 per cent. and 44 per cent. These points of inflexion were taken to mark the completion of precipitation of different sets of components. Selecting the first point of inflexion the electrodialysed enzyme was divided into two fractions, fraction I being insoluble in the presence of 35 per cent. ethanol, Γ/2 0.005 acetate, pH 4.2, and fraction II being soluble under these conditions. The two fractions were freeze-dried and paper electrophoretic studies were made, with the results shown in Figure 8. In fraction I the main protein peak is much reduced but the fraction is enriched in other components—a second peak of equal height to the first, and third and fourth peaks of smaller height can be seen. In fraction II the first peak makes up the bulk of the protein. These differences were also reflected in the physical nature of the fractions, fraction I being light brown in colour, sticky, and rich in carbohydrate, whilst fraction II was white and granular. There are marked differences also in the enzyme contents of the two fractions. Paper electrophoresis showed that fraction I contained all the protease having an isoelectric point of pH 4.2 but also some of the second protease, several amylases corresponding to the major portion of the amylase activity of this system, the sucrase, and a trace of both esterases. Fraction II contained most of the protease having an isoelectric point of pH 5.1, the viscometric protease, the two esterases, and several of the amylase components, although only a minor part of the total amylase activity.

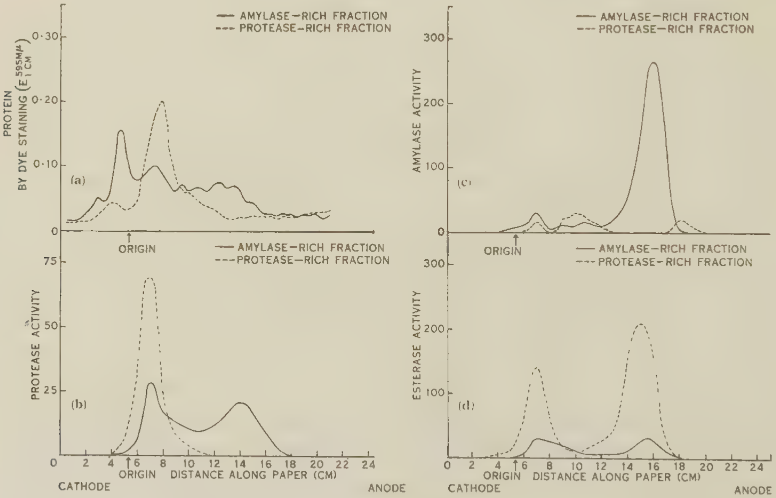

Fig. 9.—Distribution of protein and enzymes in amylase-rich and protease-rich fractions. Electrophoresis carried out in veronal buffer pH 8.6, Γ/2 0.025 for 6 hr at 7.4 V/cm. Enzyme activities in arbitrary units.

These two fractions were next subfractionated. Fraction I was split into I*A* and I*B* under the conditions of 1 per cent. protein, Γ/2 0.005 acetate, pH 4.2, −5°C; I*A* was material insoluble in 25 per cent. ethanol, and I*B* that which was soluble. Similarly, II*A* was that part of fraction II insoluble in 44 per cent. ethanol and II*B* the part which was soluble. It can be seen that the fractionation of II takes place at the second point of inflexion on the solubility curve. The four fractions were freeze-dried and examined by paper electrophoresis. It was found that fraction I*A* contained a greatly enriched protease of isoelectric point 4.2, whilst II*B* contained the protease of isoelectric point 5.1 and both esterases. The other two fractions contained protease and amylase components distributed in varying amounts. Fraction II*B* seemed to warrant further study, for it corresponded approximately to the protease-containing fraction of Crewther and Lennox (1953). In the next experiment II*B* will be referred to as the protease-rich fraction and I*A* + I*B* + II*A* as the amylase-rich fraction.

(iii) *Separation of Electrodialysed Enzyme into Protease-rich and Amylase-rich Fractions*.—From the fractionation experiments just described it can be seen that fraction II*B* consisted mainly of the major protease. To obtain a larger quantity of this material the electrodialysed enzyme was divided into two fractions by using the following conditions; protein 1 per cent., ethanol 47 per cent., – 5°C, $\Gamma/2$ 0.005 acetate, pH 4.5 These fractions were examined by paper electrophoresis in veronal buffer $\Gamma/2$ 0.025 at pH 8.6 at 7.4 V/cm for 6 hr. As can be seen from Figure 9, the protease-rich fraction contains not only the major protease, having an isoelectric point of pH 5.1, but also three amylase components and both esterases. The amylase-rich fraction contains three amylase components, corresponding to most of the amylase activity, also all of the minor protease, portion of the major protease, the catalase, and the alkaline phosphatase.

(iv) *Re-precipitation of the Protease-rich Fraction near the Isoelectric Point*.—In an attempt to purify, re-precipitation was effected at its isoelectric point, pH 5.1, under the following conditions: 1 per cent. protein, pH 5.1 acetate buffer $\Gamma/2$ 0.005, and 50 per cent. ethanol at – 5°C. The product was freeze-dried. Paper electrophoresis showed that it contained one protein component corresponding with the protease and esterase of isoelectric point pH 5.1.

Attempts were made to crystallize it from a solution containing 10 per cent. protein, acetate buffer $\Gamma/2$ 0.01, pH 5.1, and 30 per cent. ethanol, at – 5°C. On several occasions evidence was obtained of the formation of microcrystals but insufficient material was available to carry out more systematic attempts at crystallization.

(*d*) *Paper Electrophoresis Examination of Protease Crystals*

The protease crystals of Crewther and Lennox (1950), which were formed at higher ionic strength and at a higher temperature than the protease-rich fraction prepared in the present investigation, were examined electrophoretically.

The crystals (5 g) were dissolved in water, filtered to remove approximately 1 per cent. of insoluble material, and electrodialysed for 24 hr at 5°C against distilled water. After some hours a further 1 per cent. of insoluble matter formed which was filtered off and was found to have no enzyme activity on the standard substrates. The filtrate was freeze-dried to yield 1 g of white powder.

A paper electrophoretogram run on solutions of the electrodialysed crystals provided the results in Figure 10, which shows that they contained approximately 90 per cent. of a single component. This agrees with the results obtained by electrophoresis in the Tiselius apparatus and ultracentrifugation (Crewther and Lennox 1950). However, as Figure 10 shows, both proteases are present, but that with isoelectric point pH 5.1 was the more abundant. The esterase with isoelectric point pH 5.15 was also present. Similar results were obtained over a wide range of pH. As shown earlier, a sharp separation of these two proteases can be obtained by fractionation with ethanol at low ionic strength.

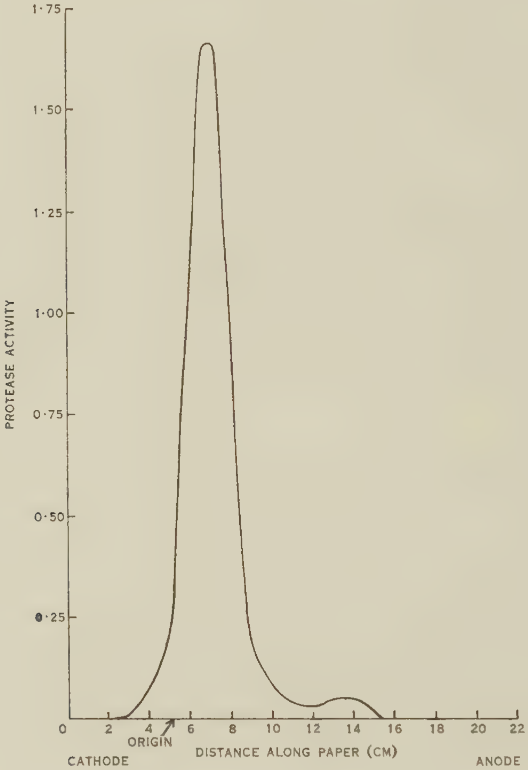

Fig. 10.—Distribution of protease in an electrophoretogram of electrodialysed enzyme crystals run in veronal buffer Γ/2 0.025, pH 8.6 for 6 hr at 7.4 V/cm. Protease activity in arbitrary units.

IV. Discussion

Salting out with neutral salts has been widely used for the fractionation and preparation of crystalline enzymes (Northrop, Kunitz, and Herriott 1948), but although this method does not damage proteins it is relatively insensitive to the specific characteristics of the various proteins in a mixture since it depends largely on the size and shape of the molecules and on the volume of water displaced. The salting-out of protein by sulphates or phosphates varies by a factor of 2 or 3 to 1 from one protein to another, and the limitations of the method were illustrated by the failure to fractionate the mould enzymes with ammonium sulphate (Crewther and Lennox 1953*b*).

In contrast with the salting-out method, the salting-in of proteins at low ionic strengths has revealed solubility ratios of the order of 10 to 1 or even 100 to 1 between different proteins. As in plasma fractionation (Cohn *et al.* 1946, 1950), ethanol has been used successfully at low ionic strengths, and at low temperatures to reduce the solubilities of the enzyme components of the *A. oryzae* culture filtrates sufficiently for their fractionation. The improved response in protein solubility to changes in ionic strength, pH, and ethanol concentration, reported for other protein mixtures to accompany a reduction in the salt concentration (e.g. Edsall 1947), is also evidenced by the solubility curves reported in the present paper. Difficulty has been experienced, however, in removing sufficient of the ionic material from the mould preparations to bring the ionic strength into the sensitive low range. Even after prolonged dialysis, the crude mould enzyme contained approximately 70 per cent. of material other than non-dialysable protein and, although partial removal was obtained by passage through a mixed-bed column of ion exchange resins, it was necessary to resort to electrodialysis for complete removal of the salts.

The change in the solubility curves of crude mould enzyme, after repeated fractionation and re-precipitation with ethanol, from a form showing minimum solubility over a wide range of pH to one having a well-defined and relatively sharp minimum (Crewther and Lennox 1953*b*) is probably attributable also to removal of ionic material.

The inflexions in the curve relating protein solubility and ethanol concentration indicated conditions for obtaining satisfactory separation of certain groups of enzymes. One of these inflexions corresponded closely with the conditions selected by Crewther and Lennox (1953*b*) for the fractionation of the mould enzyme preparatory to the production of crystals containing protease. Comparison by paper electrophoresis of the crystalline material and a protease fraction obtained from electrodialysed enzyme showed that, whereas the crystals contained a certain amount of the protease having an isoelectric point at pH 4.2 and traces of amylase, the other preparation was free of these enzymes and was, in fact, electrophoretically homogeneous, thus demonstrating the value of fractionating at low ionic strength.

The results provide further evidence of the multiple nature of the enzyme systems acting on single substrates. Thus during fractionation it has been possible to separate the two esterases previously demonstrated by paper electrophoresis (Woods and Gillespie 1953), and the amylases have been separated into groups. It is interesting to note that the traces of amylase appearing in the crystalline material correspond with one of the peaks of amylase activity shown up by paper electrophoresis. It has further been shown that, in addition to the viscometric protease and gravimetric protease of Crewther and Lennox (1953*a*) the mould enzyme contains a third protease which can be separated from the major gravimetric protease by paper electrophoresis. This third protease, which has considerable activity as measured by the azocasein method, corresponds with that having relatively low, though readily measurable, gravimetric protease activity with gelatin as substrate. It is likely therefore that the main peak of protease activity corresponds with an enzyme which acts readily on most proteins, including those lacking the aromatic amino acids.

The minor protease, having an isoelectric point at pH 4.2, apparently requires certain of the aromatic amino acids in its substrate since it acts readily on azocasein but, like chymotrypsin, is relatively inactive with gelatin as substrate. The results serve to emphasize the utility of paper electrophoresis in the study of the fractionation of complex enzyme mixtures.

The great difficulty of removing the last traces of salts from the mould enzyme suggests that the binding forces between the mould proteins and the dicarboxylic acid salts are greater than have been encountered in research with plasma proteins. The loss of enzyme activity on removal of the salts by passage of the mould enzyme through an ion exchange column was very marked. Similar observations were reported by Gilbert and Swallow (1950) for urease, α- and β-amylase, and Q-enzyme. Separation of the salts from the mould enzymes during paper electrophoresis may also have been responsible for the extreme lability of the enzymes in these experiments.

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THE SURVIVAL OF AIRBORNE MICROORGANISMS

I. EXPERIMENTS NEAR 0°C WITH SOME PSYCHROPHILIC BACTERIA

By A. D. Brown*

[*Manuscript received March* 19, 1953]

Summary

The rate of death of airborne organisms was studied for one strain each of *Achromobacter, Pseudomonas,* and *Micrococcus* at relative humidities between 45 and 90 per cent. at 0°C. For all three organisms the rates of death were least and of a similar magnitude at 65-70 per cent. R.H.

I. Introduction

The possible importance of atmosphere transport of bacteria has been realized since the end of the last century when, among others, Carnelly, Haldane, and Anderson (1887), estimated bacterial populations in the air of streets, schools, and dwellings of a Scottish town by drawing air through glass tubes lined with "Koch's Jelly" and making colony counts after incubation at room temperature. Since then interest has increased in aerial bacteriology and considerable information is now available on rapid methods of killing airborne bacteria, and on techniques and equipment suitable for estimating their numbers. It is not surprising that nearly all of this work has been done with a medical motive and under conditions closely related to those encountered under human living conditions.

On the other hand, the amount of fundamental data concerning the behaviour of airborne bacterial cells in relation to atmospheric conditions is very limited and little agreement seems to have been reached among those people who have worked in this field. Papers have been published on this and related aspects by Williamson and Gotaas (1942), Wells and Zappasodi (1942), Edward, Elford, and Laidlaw (1943), Loosli *et al.* (1943), De Ome *et al.* (1944), Rosebury (1947), and Dunklin and Puck (1948).

These authors, except Dunklin and Puck, have claimed greatest death at either high or low humidities for airborne bacteria or virus particles. Dunklin and Puck observed maximum death of certain coccal types near 50 per cent. R.H. in the presence of sodium chloride but no correlation between humidity and survival in its absence.

The present paper submits results of experiments on the behaviour of bacteria when airborne at temperatures approximating those employed in the storage of chilled beef. Three bacterial genera have been employed, all of which may be found in association with chilled beef spoilage.

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* Division of Food Preservation and Transport, C.S.I.R.O., Brisbane.

II. MATERIALS AND METHODS

(a) *Test Room*

Throughout the work a refrigerated test room was used. The volume of this room, corrected for inclusions such as brine coils etc., was 370 cu. ft and total floor area 47 sq. ft. Air was circulated by an electric fan situated in one corner of the room. This fan produced an air speed of 670 ft/min at 1 ft in front of the blades and circulated the air round the room in 10-20 sec as judged by smoke tests.

(b) *Temperature and Humidity Control*

Room temperature was maintained by cold brine circulating through side coils and control was normally effected to ± 0.2°C of the stated value. Under certain conditions, however (e.g. very cold brine), rather more variation than this was experienced. In the earlier experiments room temperature was maintained at approximately $-0.5°C$, but later this was raised to $+0.5°C$ to reduce freezing of agar plates exposed within the room.

Humidity was controlled over the range 45-90 per cent. R.H. by adjustment of brine temperature and the use of a supplementary electric heater or trays of water in the lower and upper ranges respectively. Extension of this range was not possible with the available equipment. Fluctuations in humidity of up to 3 per cent. R.H. during a run were experienced under these conditions. In early experiments humidity was measured by placing wet- and dry-bulb thermometers in front of the fan and making readings by entering the room before and after a sampling run. Readings so obtained are treated with reserve and are indicated appropriately in the results. All other measurements were made from outside the room with an instrument-reading telescope and in such cases temperature and humidity were followed throughout a run.

(c) *Introduction of Bacteria into the Test Room*

Suspensions of bacteria in the appropriate aqueous media were introduced into the test room from an atomizing device, known by the commercial name of "Phantomyst," situated about 3 ft inside the test room and about 15 in. above floor level. This instrument is classified as a nebulizer insofar as its construction causes a "refluxing" of the liquid against baffles, resulting in the emission of a spray of very fine particle size. Nebulizer performance varied with the volume of liquid employed and hence for all runs a volume of 100 ml was used. Performance also varied with temperature. Under the conditions of use when the nebulizer, at 20-25°C, was placed in the test room, a mean output of 0.55 g water per min was obtained, giving an aerosol of particles approximately 45 per cent. of whose mass had a diameter greater than 2 μ when sampled immediately in front of the outlet. Particle size was determined with the Cascade Impactor (May 1945) using slides coated with soft vaseline. Impressions in the vaseline were measured.

(d) *Air Sampling*

Air was sampled by means of a slit sampler such as described by Bourdillon *et al.* (1948). The sampler was located at floor level outside the test room and air was drawn from within through a wall pipe and two lengths of rubber hose of 1.5 in. internal diameter. The outside length (28 in.) was curved through 90° to fit over the sampler intake. The sampler was operated at a pressure reduction of 12 in. water ($\equiv$1 cu. ft/min) and the petri dish was rotated at 0.5 r.p.m.

(*e*) *Estimation of Settling*

The total number of viable organisms depositing on the floor in any experiment was estimated by exposing a number of petri dishes for the duration of a sampling run.

(*f*) *Organisms*

The organisms used in this section of the work were representative of bacterial types normally associated with the spoilage of chilled beef. All were isolated in 1933 and have been stored on nutrient agar at 0-0.5°C with transplants approximately every 6 months. The organisms have been identified generically and henceforth are referred to by their numbers in the stock culture collection of the Division of Food Preservation and Transport, C.S.I.R.O., as follows:

Achromobacter sp.	No. 483
Pseudomonas sp.	No. 451
Micrococcus sp.	No. 202

(*g*) *Media*

Media used for the growth, estimation, etc. of these and other organisms in this programme were exclusively "Difco" dehydrated media made up with glass-distilled water.

(*h*) *Experimental Procedure*

To enable rapid estimates of bacterial population to be made at the beginning of an experiment, the bacteria were grown at 20°C for periods of up to 8 days in nutrient broth (100 ml in 4-oz bottles) and the plate count was correlated with readings obtained on an E.E.L. photoelectric colourimeter using a green filter. Plate counts were made by the method recommended by Wilson (1935) for which petri dishes were incubated at 20°C for 3-4 days.

A typical run for the determination of death rate was made as follows.

The test organism was grown at 20°C for periods of 3-7 days in 100-ml quantities of nutrient broth. A measurement was made of the turbidity of the culture, which was then diluted 100-fold with the appropriate diluent. The diluted suspension was transferred to the sterile nebulizer and, together with sedimentation plates, placed in the test room. Entry of the room caused a slight disturbance of conditions but usually a stable state was re-established within 5-10 min after restarting the fan. Where possible, however, not more than 2-3 min was allowed to elapse between leaving the room and spraying the bacterial suspension, as it was sought to keep the suspension in its diluted state for as short a time as possible. This time was normally 5-7 min.

Bacteria were introduced into the air by running the nebulizer for a period determined by the turbidity of the original culture and the sampling requirements of the slit sampler. A period of 1 min was allowed to elapse between cessation of spraying and taking the first sample. Samples of 1.4 cu. ft air were taken every 7 min with a total exposure of 10 plates. These times were used in order to adhere to an earlier schedule employed with a different sampler which gave a throughput of 0.7 cu. ft/min for the same air velocity at the slit (i.e. 12 in. water pressure reduction).

At the conclusion of sampling the fan was stopped, the room again entered, and sedimentation plates covered and removed along with the nebulizer. The mean time at which settling plates were covered was recorded and regarded as the end of a run. All petri dishes were incubated at 20°C for 3 or 4 days (depending on the organism) and then counted. Counts so obtained were treated as described below.

III. Expression of Results

Rate of loss of cells from the air is expressed in terms of changes of air per hour and represented by the symbol K as defined and calculated by Bourdillon *et al.* (1948). This symbol, expressed as hr^{-1}, may also be defined as the number of viable particles disappearing from the air from any cause per unit time, per unit volume, per unit concentration, and this definition may be shown to agree with that of Bourdillon *et al.*

Sedimentation rate is expressed in similar units by the symbol K_s which is calculated from the equation

$$K_s = \frac{\Delta S}{\Delta N} K,$$

and not, as stated by Lidwell (1948), from the equation

$$K_s = \frac{\Delta S}{\Delta N} \cdot \frac{V}{A} \cdot K,$$

where the symbols are Lidwell's. Lidwell (personal communication) has recently indicated his agreement with the former equation. When K_s is calculated in this way, the death rate, K_d, may be calculated in the above units, from the equation

$$K_d = K - K_s.$$

In all cases K has been calculated statistically with the assumption that deviation remained constant over a run. Similarly comparisons of results have been made statistically and statements of no difference are made on this basis.

IV. Results

(a) Mechanical Factors

(i) *Removal from Walls.*—A preliminary survey was made to assess the importance of removal by air circulation of bacteria from the walls of the test room. The results of this survey showed that when a broth suspension containing 10^8-10^9 bacterial cells was smeared over 8 sq. ft on one wall and the fan directed at this wall, disturbance of cells became very significant. On the other hand, when the same number of cells was distributed over the four walls (2 sq. ft per wall) and the fan left in its normal position, no increase in the number of airborne cells could be detected in the room up to 90 min after making the smears.

(ii) *Deposition on Walls.*—The rate of impingement of cells on the walls was measured by attaching to the walls microscope slides coated with nutrient agar. Impingement on the ceiling was not measured because it was assumed to be negligible and because the excessive time needed for the attachment would have resulted in marked disturbance of the atmospheric conditions.

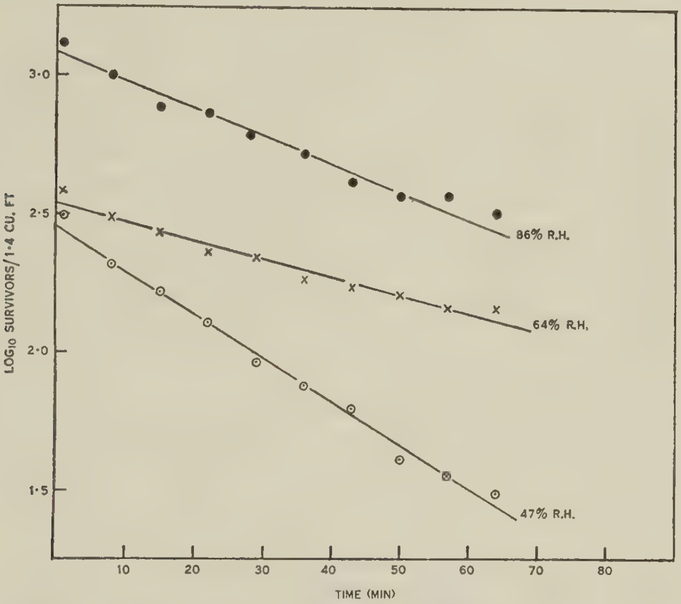

Fig. 1.—*Achromobacter* sp. (483). Regressions of log survivors *v.* time at three levels of humidity.

Mean results of two such runs at 87-89 per cent. R.H. with *Pseudomonas* No. 451 are:

$$K = 2.57 \qquad K_s = 0.10, \qquad K_w = 0.037,$$

where K_w = the rate of deposition on the walls expressed in the same units as the other K values.

(iii) *Sampling Efficiency.*—Evidence was sought that all viable cells delivered to the test room could be accounted for. Accordingly, on a number of runs the bacterial suspension to be used was treated for 1 min in a Waring Blendor to break up clumps, and plate counts were made on the treated suspensions. Treatment in the Blendor was necessitated by the action of the nebulizer, which itself breaks up clumps and delivers an essentially monocellular aerosol. On the basis of a delivery from the nebulizer of 0.54-0.55 g water per min and the particle size characteristics already described, agreement to within 10 per cent. was usually obtained with the number of cells estimated by air sampling.

(*b*) *Effect of Diluent*

Rates of death of *Achromobacter* No. 483 at 65-72 per cent. R.H. were similar when either nutrient broth or tap water was the diluent used in preparing suspensions. Tap water was used subsequently for experiments with all three organisms. Such determinations in the 65-70 per cent. R.H. region are accompanied by disadvantages which will become apparent later.

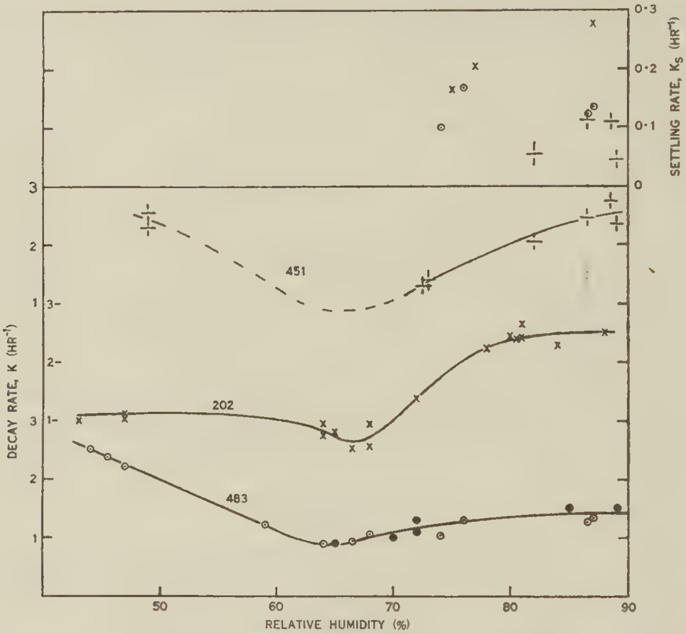

Fig. 2.—*K*/humidity relationships of *Achromobacter* No. 483, *Micrococcus* No. 202, and *Pseudomonas* No. 451. ●, Room entered for humidity readings.

(*c*) *Effect of Age of Culture*

Using *Achromobacter* No. 483 and *Micrococcus* No. 202, the effect on *K* of age of culture beyond the logarithmic growth phase was investigated between 63 and 72 per cent. R.H. No differences in death rates were found for cultures aged 3-7 days at 20°C. It will be seen from later results in this and the following paper that the selection of the above humidity range to test an age effect was not a happy one, but analyses of results of all three organisms at all humidities tested failed to reveal any variations that could be associated with differences in age within the range employed.

(*d*) *Effect of Relative Humidity*

Over the available humidity range, death of the three airborne organisms was always a simple logarithmic process and typical regressions of log survivors *v.* time are shown for the *Achromobacter* No. 483 in Figure 1.

The relationships between *K*, K_s, and R.H. of all three bacteria are presented in Figure 2, from which it will be seen that, for 483 and 202, *K* values were least and of like magnitude at humidities in the region of 65-70 per cent. R.H. Similar behaviour by 451 may be inferred, although the absence of points between 49 and 73 per cent. R.H. prevents a definite conclusion.

V. DISCUSSION

The generic differences in sensitivity to changes in humidity shown in Figure 2 are of some interest and tend to discourage generalization about the effect of humidity on survival of airborne bacteria. It may be noted that the results obtained with either the *Achromobacter* (483) or the *Micrococcus* (202), considered separately, could be used either in support of or in opposition to the schools which have claimed either a positive (Williamson and Gotaas 1942; De Ome *et al.* 1944; Lidwell and Lowbury 1950) or a negative (Wells and Zappasodi 1942; Rosebury 1947) correlation between death rate and humidity.

One aspect of behaviour common to the three bacteria and which may lead to a generalization, however, was the occurrence of minimal death rate in the 65-70 per cent. R.H. region. One is tempted to think that two levels of humidity may prove to be potentially lethal to airborne bacteria over the range 45-90 per cent. R.H. and that the survival/humidity relation of any species is determined by its sensitivity at each of these levels. Further evidence on this concept is presented in the following paper.

VI. ACKNOWLEDGMENTS

The writer is indebted to Mr. A. Howard for advice on physical aspects of this work, and to Mr. F. Grau for technical assistance.

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THE SURVIVAL OF AIRBORNE MICROORGANISMS

II. EXPERIMENTS WITH *ESCHERICHIA COLI* NEAR 0°C

By A. D. Brown*

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Summary

The humidity/death rate relationships of two types of airborne *Escherichia coli* were studied at approximately 0.5°C over the range 45-100 per cent. relative humidity. Support was obtained for previous findings with psychrophilic bacteria of minimal death rates at about 70 per cent. R.H.

Evidence suggests that after spraying into the air from an aqueous suspension, bacterial cells come rapidly into equilibrium with the atmosphere and that measured death rates are associated with these equilibrium conditions.

At least two separate lethal mechanisms operating near 50 per cent. and 90 per cent. R.H. respectively seem to be potentially available for the destruction of airborne bacteria. For *Esch. coli*, at least, the lower-humidity mechanism is probably a direct water activity effect. The high-humidity mechanism seems to be neither a water activity effect nor a "cold shock" reaction.

Death of *Esch. coli* at low humidities was not affected by the age of the cells, but at high humidities death was most rapid with young cultures.

Differences in behaviour at high humidities were observed between rough and smooth, and aerated and anaerobic cultures.

I. INTRODUCTION

It was shown previously (Brown 1953) that certain bacteria when airborne at a temperature near 0°C are likely to have a minimal death rate in the region of 70 per cent. relative humidity (R.H.). The bacteria used were capable of growth at 0°C. Therefore experiments were made near 0°C using an organism with mesophilic temperature requirements. Although it has no significance in chilled meat storage, *Escherichia coli* was selected for this investigation as it has the advantages of a well-studied physiology and has been used in air disinfection studies at higher temperatures. In the present paper attention is also directed towards the elucidation of factors that may affect the sensitivity of a bacterial cell to atmospheric conditions.

II. MATERIALS AND METHODS

The same equipment as described in the previous paper (Brown 1953) was used, but slight modifications in technique were made, as described.

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(*a*) *Humidity and Temperature Control*

A vessel of water heated electrically by an immersion unit was substituted for the trays of water in the high humidity range. This change facilitated a more continuous selection of humidities and permitted extension of the range to 100 per cent. R.H. The immersion heater was also used to raise the humidity 10-15 per cent. during the course of some experiments.

In later runs at 90 per cent. R.H. room temperature was raised to 1°C to avoid freezing of the wet thermometer bulb. This change had no measurable effect on death rate.

(*b*) *Sampling*

Because of evidence of an early change in death rate in some cases, the sampling schedule was modified so that the first six exposures were of 30 sec duration at intervals of 1 min. These plates were rotated at 2 r.p.m. In the later work at 90 per cent. R.H. all plates were exposed for 30 sec and rotated at 2 r.p.m. to a time schedule most suited to the death rates encountered. Incubation was at 37°C for 18-22 hr.

(*c*) *Organisms*

Esch. coli, types 1 and 2, were used. The organisms were grown at 37°C in the following ways:

(1) As standing cultures in tubed nutrient broth (6-8 ml) for 18 hr. These cultures were also used as inocula.

(2) As surface cultures on nutrient agar for 18 hr.

(3) In nutrient broth (60 ml) aerated by the principle described by Skerman, Lack, and Millis (1951).

(4) Anaerobically in nutrient broth (6 ml) in evacuated glass ampoules.

Standing tubes and agar cultures were inoculated with approximately 10^6 cells. In the other cases the inoculum is given with the appropriate results.

In order to observe time schedules etc., it frequently became necessary to store a culture for up to 4 hr at -0.5°C before use. This treatment was found to have no effect on subsequent results. Broth cultures were harvested by centrifugation and resuspended in an equal volume of sterile glass-distilled water. The turbidity of this suspension was determined and a calculated volume diluted to 100 ml with sterile glass-distilled water. For some types of experiment turbidity was measured in the original broth culture. Agar cultures were dispersed into glass-distilled water (6 ml) and thence treated identically with the broth-grown cells. Suspensions obtained in this way were sprayed for 1 min into the test room.

In the course of the work it was found that frequently, but not invariably, the death rate of airborne cells was not a simple logarithmic function. In such cases a high death rate was obtained in the first 10-20 min of a run, after which the rate decreased to a value which was usually the same as that obtained when no change in slope was observed. An expression of the initial rapid death as a rate was difficult as in most instances plots of log survivors against time were best joined by a smooth curve which merged into the "normal" linear regression. During the early part of the investigations the following observations concerning this change in slope were made:

(1) The change in slope could not be associated with any change in the atmospheric conditions in the test room.

(2) Of the organisms examined in this and the preceding paper (Brown 1953) the type 2 and one type 1 strain of *Esch. coli* were the only ones in which the phenomenon was observed.

(3) Conditions which caused an increase in "normal" death rate also caused an increase in the initial high death rate.

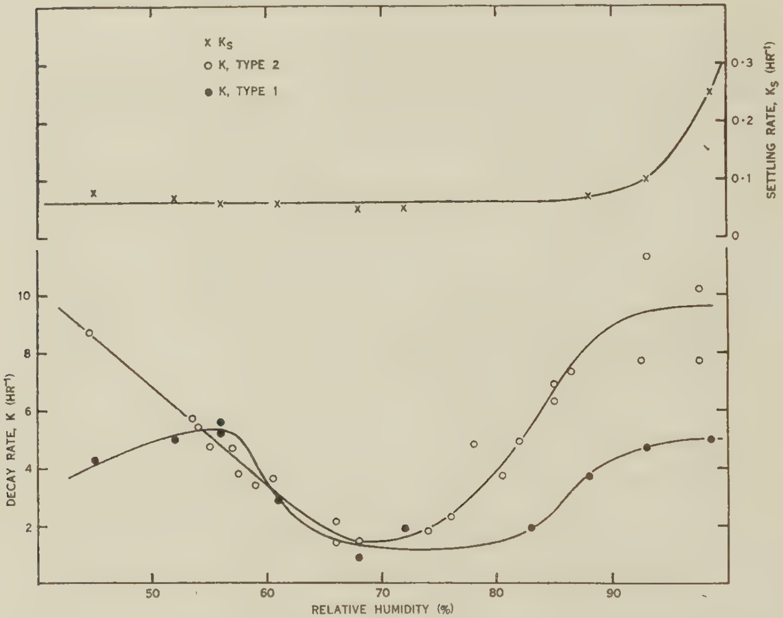

Fig. 1.—*Esch. coli*, types 1 and 2. Decay rate (K) and settling rate (K_s) *v.* humidity, relationships of standing broth cultures.

It was subsequently realized that the type 1 strain which gave an initial high death rate, also produced a slightly rough mutant, whereas a second type 1 strain, which showed only a smooth-mucoid transformation, always gave logarithmic death.

A little later in the programme the type 2 organism at high humidities began to give atypical results, which included complete departures from logarithmic death for the duration of an experiment. At this stage it was realized that rough colonies were present and subseqently at least three different variants were isolated, ranging from smooth to very rough. The smoothest variant was selected for extension of the investigations. While it remained smooth, logarithmic death was always obtained (at high humidities) and, conversely, departures from a straight line were always associated with colony heterogeneities. On the other hand, colony heterogeneities were not always associated with non-logarithmic death. Departures with this strain were most commonly observed in old cultures (e.g. 48 hr).

Values of K which follow represent the "normal" values in all cases. The calculation of K_s was complicated when non-logarithmic death occurred and values given are derived from runs in which the change in slope was least significant.

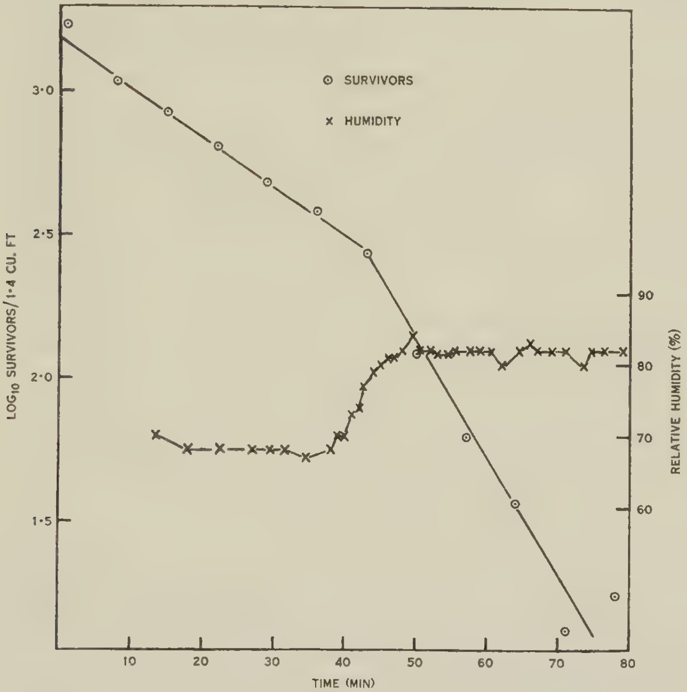

Fig. 2.—*Esch. coli*, type 2. Effect on survival of increasing humidity from 68 to 82 per cent. R.H. Standing tube culture (cf. Fig. 1).

III. Results

(*a*) *Effects of Relative Humidity*

(i) *Death/Humidity Relations*.—The relations between decay rate (K), settling rate (K_s), and relative humidity are shown in Figure 1 for *Esch. coli* types 1 and 2. The occurrence, in both strains, of minimal death rate in the 70 per cent. R.H. region and the magnitude of K at this minimum are both features of similarity with the psychrophilic bacteria already described. In view of the relatively small number of points obtained the location of the maximum in the type 1 curve should not be regarded too rigidly. Because of the greater magnitude of the effects on type 2, this strain was used in the extension of the work.

(ii) *Reversibility of Desiccation*.—In experiments in which humidity was raised some 40 min after delivery of the bacterial aerosol, it was found (Figs.

2 and 3) that desiccation of the cells was readily reversible (as measured by death rate) and that the measured death rates were associated with equilibrium conditions.

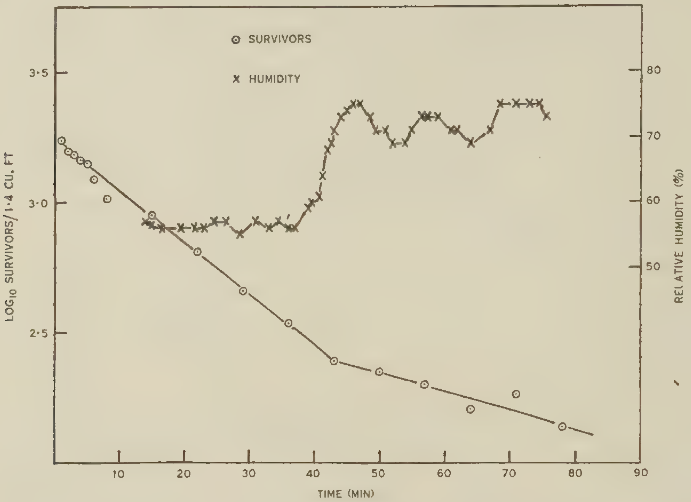

Fig. 3.—*Esch. coli,* type 2. Effect on survival of increasing humidity from 56 to 73 per cent. R.H. Agar surface culture (cf. Fig. 1 and Section III (*b*) (i)).

(*b*) *Effects of Growth Conditions and Changes in Physiology*

(i) *Growth on Agar Surfaces.*—In further experiments in which growth conditions were varied, cells grown on agar surfaces were found to have the same death rates below 70-75 per cent. R.H., but were much more resistant at high humidities than broth-grown cells (Table 1).

Table 1

ESCHERICHIA COLI TYPE 2. COMPARISON OF DEATH RATES OF BROTH- AND AGAR-GROWN CELLS WHEN AIRBORNE AT HIGH HUMIDITIES

Relative Humidity (%)	$K_d(\mathrm{Hr}^{-1})$*	
	Broth-grown	Agar-grown
75	1·6	1·2
80	4·0	1·6
85	6·5	2·1
90	9·0	3·5
95	9·4	5·2

* Values for K are interpolated from Figure 1 for the broth-grown cells and from a similar curve (not shown) for the agar-grown cells.

(ii) *Effect on Survival of Aeration During Growth.*—The foregoing results suggested a possible implication of oxygen in determining sensitivity at high humidities. Accordingly a number of comparisons was made at high and low humidities using cells grown for 18 hr in four different ways ranging in effect from complete anaerobiosis to full aeration. The results of this comparison are given in Table 2, from which it is seen that, again, little or no effect was apparent at low humidities, while at 90 per cent. R.H. greatly increased resistance was associated with aeration during growth.

Table 2

EFFECT OF OXYGEN DURING GROWTH ON SURVIVAL OF AIRBORNE *ESCH. COLI* TYPE 2

Growth Conditions (18 hr at 37°C)	Inoculum (Approx. No. Cells)	Turbidity (Arbitrary Units)	Relative Humidity (%)	K (Hr^{-1})	Standard Deviation
Anaerobic broth	10^6	2-3	90	13·1	±0·76
			52	5·8	±0·90
Standing tubed broth	10^6	3-10	90	8·8	—
			52	6·6	—
Aerated broth	5×10^6	27-31	90	2·8	±0·06
			56-57	3·8	±0·07
Agar surface	10^6	—	90	3·6	—
			56-57	4·4	—

(iii) *Effect of Age of Culture.*—The above results could be attributed either to a direct effect of oxygen on sensitivity of the cell or to an indirect effect on the physiological age of the culture. To test this latter possibility additional runs were made in which comparatively high death rates ($K_d = 11\text{-}17$) were found at 90 per cent. R.H. for young (4-8 hr) aerated cultures and comparatively low death rates ($K_d = 3\text{-}4$) were found at the same humidity for old (2 days) anaerobic cultures. These findings were extended by an examination of a complete growth cycle of the organism, grown anaerobically and aerobically for examination at 90 per cent. R.H. and aerobically only for examination near 50 per cent. R.H.

At the lower humidity, death rate was independent of the age of the culture.

The results at the high humidity are illustrated in Figure 4. From this it appears probable that the maximal death rate occurred during the transition from lag to logarithmic growth phase, or else very early in logarithmic growth. Because of the uncertainty in locating this maximum it would be unwise, at present, to comment on the identity or otherwise of anaerobic and aerated cultures in this part of their growth cycle. It will be noted, however, that these two types of cells showed quantitative differences after the cessation of logarithmic growth, the airborne anaerobic cells dying quite rapidly until comparatively late in their growth cycle.

(iv) *The Smooth-Rough Change.*—Because of the previously described (Section II) association of rough colonies with non-logarithmic death, a very rough strain of *Esch. coli* type 2 was examined at 90 per cent. R.H. over a complete growth cycle. The findings are summarized in Table 3, from which it will be seen that, whereas resistance of the smooth culture increased rapidly during the logarithmic growth phase (see also Fig. 4), resistance of the rough culture was constant over that phase but subsequently increased to the same level as that of the smooth.

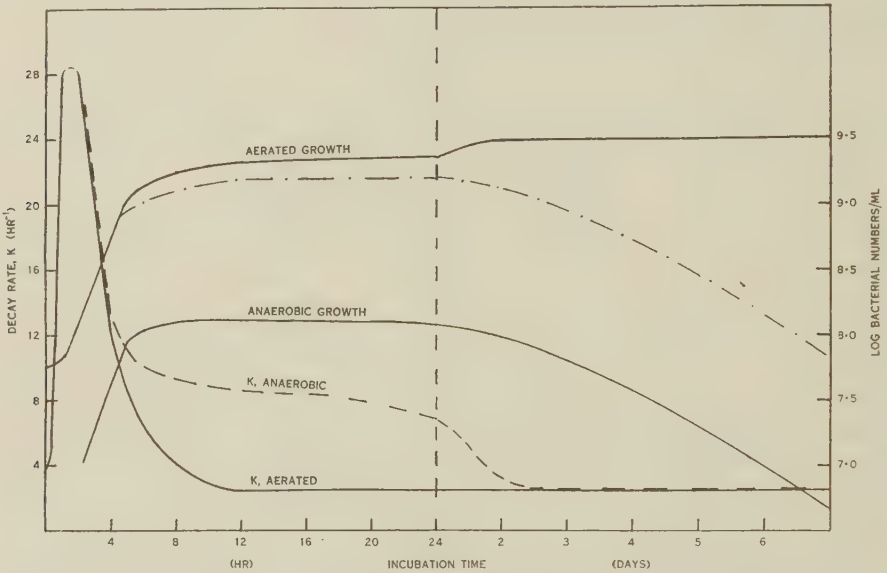

Fig. 4.—*Esch. coli*, type 2. Decay rate (K) *v.* culture age, relationship at 90 per cent. R.H. This figure is intended to depict the relationship of death of airborne cells to their phase of growth, not necessarily to their absolute age. The continuous aerated growth curve represents bacterial mass expressed as $\log_{10}$ number of cells. The broken line is the approximate course of the aerated viable count. The anaerobic growth curve also represents viable count. Anaerobic growth was followed by means of a series of ampoules inoculated simultaneously and broken as required for air sampling and viable count determinations. Inocula were: aerated growth, 3 ml standing culture (10^9 cells); lag phase (aerated), 10^{10} cells (centrifuged); anaerobic growth, 0.05 ml standing culture (2×10^7 cells).

(*c*) *The Lethal Mechanisms*

(i) *Death Rates in Aqueous Solutions.*—Survival was measured at 0.5°C in aqueous solutions of glycerol at concentrations corresponding to the humidities at which high and low death rates were observed in air (see Fig. 1). The results of these measurements are given in Table 4 and imply that the death rates in air at humidities below 70 per cent. R.H. are very likely due to a simple water activity effect. The rates of death in solution at 93 per cent. R.H., however, which were subsequently confirmed with solutions of NaCl, KCl, and sucrose, were markedly different from the values obtained in air and suggest that an explanation involving factors other than water activity should be sought for the high death rates at high humidities.

(ii) *Cold Shock.*—The close relationship found between culture age and resistance at high humidities suggested that the lethal mechanism in this zone may involve a "cold shock" such as described by Hegarty and Weeks (1940). It was felt that the foregoing results of survival in solutions were sufficient to discount prolonged exposure to cold as a lethal process, but the effect of the sudden initial cooling during atomization remained to be considered. Accordingly, aerated *Esch. coli* cultures at the beginning and middle of their logarithmic growth phases were cooled rapidly from 37°C by making 100-fold dilutions with nutrient broth and quarter-strength Ringer's solution maintained at 0 and 0.5°C. Viable counts were made immediately before dilution, immediately after dilution, 5 min after, and at intervals of 10 min from 15 to 35 min after dilution. The results of these measurements may be summarized as follow.

TABLE 3

ESCH. COLI TYPE 2. DEATH RATES AT 90 PER CENT. R.H. OF ROUGH AND SMOOTH STRAINS

Growth Phase (In aerated culture)	Approximate K_d (Hr^{-1})	
	Smooth	Rough
Early logarithmic	28-30	18-20
Late logarithmic	8-10	18-20
Stationary and decline	2·5	2·5

TABLE 4

DEATH RATES* OF *ESCH. COLI* IN AIR AND GLYCEROL SOLUTIONS AT 0.5°C

Relative Humidity (%)	Death Rate, K_d (Hr^{-1})	
	Air	Solution
93	8-10	0·32-0·39
70	1·2±	0·6-1·8
55	5·2±	4·0-5·0

* Death rates in solution were determined by making five viable counts at intervals of 15 min. Glycerol concentrations were obtained from International Critical Tables (1928).

Early log phase cultures suffered destruction ranging from 0 to 40 per cent. immediately on cooling, but underwent no further change on holding at the lower temperature. Mid-log phase cultures suffered no measurable destruction on cooling.

Additional results were obtained in which identical death rates were observed at 90 per cent. R.H. for mid-log phase cultures atomized from (*a*) suspensions at 20-25°C and (*b*) suspensions allowed to equilibrate with the test atmosphere (*c.* + 1°C).

(iii) *The Possibility of Specific Toxic Concentrations of Endogenous Substrates.*—At neither 50 nor 90 per cent. R.H. was any difference found between the death rates of unwashed cells with high endogenous respiration and cells washed with phosphate buffer to have little or no endogenous respiration.

IV. Discussion

Before discussing individual aspects of the foregoing results, it is important to consider two possible complications arising from the fact that the investigation was conducted near the freezing point of water.

The first of these is the possible effect on sedimentation figures of freezing in the settling plates. It was usual for some plates to freeze at humidities of about 70 per cent. and all plates invariably froze at 50 per cent. R.H. The K_s/humidity curve of Figure 1, which is flat over the range where freezing occurs, and a comparison of counts of frozen and unfrozen plates from many experiments, have provided evidence that freezing of the medium does not measurably affect the survival of cells which have deposited on it.

The second possibility is that the airborne cells themselves may freeze. It will be apparent from the outset that this question does not arise in the experiments at 90 per cent. R.H. in which the wet bulb temperature of the room remained above 0°C. The existence of ice crystals in the cell is also impossible after thermodynamic equilibration with the atmosphere. The possibility of freezing is therefore limited to the initial period of desiccation of the particles and this time may be shown to be very short in relation to sampling intervals. The ability of ice to form during this period will, of course, be determined by the wet bulb temperature of the room, heat transfer rates, and the freezing point of the cell, which, for cells grown in nutrient broth has been placed between −0.2 and −1.7°C (W. J. Scott, personal communication).

The time of evaporation of external water from the bacteria-carrying particle may be gauged from a theoretical equation of Langmuir (1918) as used by Dunklin and Puck (1948). From this equation the author has calculated a time of 0.026 sec at 0°C and 80 per cent. R.H. for spherical droplets of 2 μ diameter containing a rod-shaped cell 2 by 1 μ, to lose all extra-cellular water, assuming no change in evaporation rate with change in shape of the droplet and no diffusion of solutes from the cell to the external water. In actual fact the time is probably somewhat longer than this owing to local rises in humidity caused by the aerosol, but not so much longer as to affect the validity of the dependent conclusions.

It may be inferred from experimental results that the complete period of equilibration (i.e. including loss of intracellular water) is also quite short. If this period were of sufficiently long duration, relative to sampling intervals, the death rate over the first part of a run at low humidity would be expected to be represented by a curve. The slope of the curve at any time would then be equal to the equilibrium K corresponding to the higher humidity to which the cell had dried out at that time. Nothing of the kind has been observed, although it must be presumed to happen over the period of equilibration. The high initial death rate of *Esch. coli* bears no relationship to "normal" death rates at any higher humidity.

Similarly, Figures 2 and 3 show that the regressions of log survival against time for each humidity were best joined by straight lines. The rate of readjustment of the cell to the new condition therefore was again short in relation to the intervals between samples (in this case 7 min). It may be concluded therefore that, under conditions such as described, airborne bacterial cells come into thermodynamic equilibrium in less time than is measurable by death rate response. This conclusion differs from that of Robertson and Lester (1951), who contend that "desiccation" at 15 per cent. R.H. may continue for up to 20 hr.

The effect of humidity on the survival of both types of *Esch. coli* is in general agreement with the trend of results in the preceding paper (Brown 1953). It will be noted that in spite of the similarities in minimal K values of all the organisms examined, death rate (K_d) is still the major component of K. This conclusion is supported by additional, unquoted, sedimentation figures obtained by the use of a direct microscopical method and by experiments with bacterial spores.

While the differences between agar and standing broth cultures (Table 1) may be explained in the simple terms of differences in physiological age, the additional difference between aerated and anaerobic cultures shown in Figure 4 may also be implicated. At present no explanation is offered for this difference.

The immediate cause of the non-logarithmic death rates has not been discovered. Certain associations have been observed but do not cover all occurrences of the phenomenon. A sufficiently high proportion of the rough strain in a late logarithmic culture at high humidities (Table 3) would produce such a change in slope but this is an isolated case.

Some tentative conclusions may be drawn regarding the factors affecting death of airborne cells. At least two distinct mechanisms seem to be potentially available for the destruction of cells. In *Esch. coli* the mechanism operating near 50 per cent. R.H. is very likely a simple water activity effect. Only negative information is available regarding the high-humidity mechanism, which on the evidence is neither a water activity nor a cold shock reaction nor is it affected by washing the cells with phosphate buffer. The results of the cold shock experiment are not intended to be quoted in opposition to those of Hegarty and Weeks (1940), as in all probability the discrepancies may be explained by the different strains of organism they used.

VI. ACKNOWLEDGMENTS

The writer is indebted to Mr. A. Howard for advice on physical aspects of this work, and to Mr. F. Grau for technical assistance.

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THE EFFECTIVENESS OF ANTIBIOTICS AGAINST SOME BACTERIAL PLANT PATHOGENS

By J. H. E. MACKAY* and JANET N. FRIEND*

[*Manuscript received December* 16, 1952]

Summary

In vitro tests of six purified antibiotics against bacterial pathogens of dwarf bean, tomato, and *Brassica* spp. showed that streptomycin and aureomycin were slightly more effective than terramycin, and all three were more effective than penicillin, bacitracin, and chloromycetin.

Corynebacterium michiganense (Smith) Jensen, the only Gram-positive organism tested, was the most sensitive, and the only organism inhibited by all six antibiotics within the concentrations used.

I. INTRODUCTION

The possibility of using antibiotic materials for the control of plant diseases has received increasing attention in recent years. Reviews of the subject have been published by Ark (1949), Wiendling (1950), Grossbard (1951), and Anderson and Gottlieb (1952).

The minimum concentrations of the various antibiotics required to inhibit growth of bacteria pathogenic to man have been adequately collated and reported. Corresponding data for plant pathogens are not readily available.

Whiffen (1950) reported *in vitro* tests with actidione against plant-pathogenic fungi. Gilliver (1946) studied the effects of 13 antibiotic substances on 33 bacterial and fungal plant pathogens, and concluded that some of the materials tested might be useful in the control of plant disease. All the materials used in the present investigation, except penicillin, have been developed since Gilliver's work was reported.

In order to select antibiotics which might be of use as chemotherapeutants against bacterial diseases, *in vitro* studies were done using purified crystalline materials. Since the most important bacterial diseases under Australian conditions are those of tomato, dwarf bean, and members of the genus *Brassica*, work was confined to pathogens of these plants.

II. MATERIALS AND METHODS

Preliminary work using the cylinder-plate method of assay gave results which were not readily comparable, and the method was discarded in favour of the tube dilution method. Since some of the antibiotics inhibited growth at very low concentrations (< 0.25 μg/ml) only a limited dilution range was used, the most concentrated solution containing 50 μg/ml.

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All test organisms grew satisfactorily at 27°C in nutrient broth + 2 per cent. glucose. Assays were done in triplicate and determined turbidimetrically after 48 hr incubation. The end-point adopted was that dilution at which no visible growth occurred. One per cent. of a 24-hr broth culture of the test organism was used as inoculum.

Antibiotic solutions containing 50 μg/ml were made up in sterile broth and diluted to give the final concentration before tubing. An aliquot of 10 ml was placed in each tube. In no instance was broth containing antibiotic autoclaved. To check sterility a series of uninoculated dilutions was included in each assay.

The test organisms are listed in Table 1 and the antibiotics used were:

(1) Streptomycin, calcium chloride : Glaxo, batch No. 916; 700 units/mg

(2) Aureomycin hydrochloride : Lederle, lot No. 4474-10486

(3) Terramycin hydrochloride : Pfizer, lot No. 507265

(4) Chloromycetin : Parke Davis & Co. (Sydney), AL 154 B

(5) Bacitracin : C.S.C. Pharmaceuticals, lot No. 51050305

(6) Penicillin, potassium benzyl : Cwth. Serum Labs. (Aust.), 1600 units/mg.

Throughout this work all results were confirmed by repetition. In the first instance a series of dilutions containing 50, 25, 10, 5, 1, 0.5, and 0.25 μg/ml was set up. After completion of this assay either of two courses was followed to confirm results or obtain further information.

(1) If total inhibition occurred at 0.5 or 0.25 μg/ml, or there was no inhibition at 50 μg/ml, the assay was repeated at these concentrations.

(2) If, in the more widely separated intermediate steps of the series, inhibition occurred at one dilution and growth at the next, a series of intermediate dilutions was set up to establish a definite end-point between the two concentrations.

III. Results and Discussion

The results of this work are presented in Table 1. Streptomycin and aureomycin were the most effective antibiotics against the organisms tested. Terramycin was never more effective than either of these, but was equally as effective in several instances. Bacitracin and penicillin were, within the range of concentrations used, effective against *Corynebacterium michiganense* (Smith) Jensen only, and must be considered inferior to the other materials as possible chemotherapeutants. Chloromycetin inhibited three of the six pathogens tested, but because of the relatively high concentrations required it compares unfavourably with streptomycin and aureomycin.

C. michiganense, the only Gram-positive organism tested, was more sensitive to antibiotics than any other pathogen. Ark (1949) demonstrated that seed treatment with streptomycin was an effective means of controlling bacterial canker of tomato caused by this organism.

This work has shown that all the seed-borne bacterial pathogens investigated are inhibited *in vitro* by either streptomycin or aureomycin in very low concentration, and it is feasible that these materials may prove effective seed treatments for control of the diseases caused by them.

TABLE 1

RESULTS OF ASSAYS OF SIX ANTIBIOTICS USING VARIOUS PLANT PATHOGENIC BACTERIA AS TEST ORGANISMS

Organism	Disease Caused	Minimum Concentrations for Inhibition (μg/ml)					
		Streptomycin	Aureomycin	Terramycin	Chloromycetin	Bacitracin	Penicillin
Corynebacterium michiganense (Smith) Jensen	Bacterial canker of tomato	<0·25	0·5	2·0	6·0	<0·25	1·0
Xanthomonas vesicatoria (G. & K.) Dowson	Bacterial spot of tomato	2·0	0·5	2·0	50·0	>50·0	>50·0
X. phaseoli (Smith) Dowson	Common blight dwarf bean	0·5	0·5	4·0	25·0	>50·0	>50·0
Pseudomonas syringae van Hall	Leaf blotch of dwarf bean	0·5	2·0	2·0	>50·0	>50·0	>50·0
Ps. medicaginis v. *phaseolicola* (Burk.) Dowson	Halo blight of dwarf bean	<0·25	1·0	1·0	>50·0	>50·0	>50·0
X. campestris (Pammel) Dowson	Black rot of *Brassica* spp.	2·0	<0·25	2·0	>50·0	>50·0	>50·0

Colasito *et al.* (1952), using streptomycin sulphate (855 units/mg), found that much greater concentrations than those reported here were required for inhibition of *Xanthomonas phaseoli* (Smith) Dowson and *X. vesicatoria* (G. & K.) Dowson. de Ropp (1949) demonstrated a wide range in sensitivity between strains of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. to various antibiotics. The strain most sensitive to streptomycin was inhibited by 0.2 μg/ml while 50.0 μg/ml were required to inhibit the most resistant. Strain difference and the streptomycin compound used may account for the discrepancy between Colasito's results and those obtained in this work. The demonstrated variability in strain reaction complicates the problem of control and it may be necessary to vary treatment according to the bacterial strain present.

To be useful as a means of control for the diseases concerned in this work, any treatment should be capable of controlling all pathogens of a single host at about the same concentration. The results presented in Table 1 show that streptomycin fills this condition for dwarf beans and aureomycin for tomatoes.

Work on various aspects of disease control is being continued with these antibiotics.

IV. ACKNOWLEDGMENTS

The authors wish to express their appreciation to Professor W. L. Waterhouse, University of Sydney, Mr. H. Freeman, Victorian Department of Agriculture, and Mr. W. S. Sutton, New South Wales Department of Agriculture, for supplying cultures used in this work, and to Mr. G. Anderson, Commonwealth Serum Laboratories, for providing the potassium benzyl penicillin and for advice in the early stages of the work.

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REGENERATION AND THE MOULTING CYCLE IN *BLATTELLA GERMANICA* L.

I. SINGLE REGENERATION INITIATED DURING THE FIRST INSTAR

By A. F. O'Farrell* and A. Stock*

[*Manuscript received March* 2, 1953]

Summary

The effects of removal of the left metathoracic leg of *Blattella germanica* at the proximal autotomy plane, i.e. between trochanter and femur, are described from operations on animals at known ages in the first instar, at temperatures of 25 and 30°C, 70-80 per cent. R.H. Either a completely differentiated regenerate or an undifferentiated papilla, but *never* an intermediate, appears at the first moult after operation. Where a papilla is produced the complete regenerate appears at the second moult after operation. Ecdysis is not delayed by production of a papilla, but is delayed by production of a complete regenerate. The interval between the operation and the first moult then approximates to the duration of the first instar of controls, being greater with operations very early in the instar and less with later ones.

There is a "critical period" in the first instar, before which operation gives rise to a regenerate with delay in the first ecdysis, and after which it results in production of a papilla without delay in ecdysis. Delay in the first ecdysis resulting from regeneration appears to be compensated by a subsequent speeding up of the moulting cycle, the ages of operated animals at their fourth ecdysis being approximately the same as those of controls. Hypotheses are discussed relating these results to endocrine control of the interaction between regeneration and the moulting cycle. The possibility of using regenerating *Blattella germanica* in experimental work on moulting and metamorphosis is suggested.

I. INTRODUCTION

Although one of the earliest studies on regeneration of an insect leg was made by Brindley (1897, 1898) with the cockroach *Blatta orientalis*, the Blattidae have since received little attention from workers in this field. Morphological features and comparative growth rates of regenerating and normal legs have been studied by Woodruff and Seamans (1939) in *Blattella germanica*, and by Voy (1949, 1951) in *Blatta orientalis*. A general, but irregular, increase in the total number of moults undergone by regenerating animals was suggested by Zabinsky (1936) for *Periplaneta*, and confirmed by Seamans and Woodruff (1939) for *Blattella*. Much work on Phasmidae and Mantidae, e.g. Przibram (1931), and the recent study by Luscher (1948) on *Rhodnius* (Hemiptera) suggest that, at the first ecdysis after an operation leading to regeneration of a leg or part of a leg, the regenerate reaches a level of differentiation largely dependent on the time elapsing between the operation and the next ecdysis.

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Voy (1951), however, reported that *Carausius* will produce a differentiated but incomplete regenerate if operated on before a certain critical stage in an instar, but at most a blastema within the old stump if operated on after this stage. However, little attention seems to have been paid to the relationship between regeneration and the moulting cycle. The present paper describes some investigations on *Blattella germanica* indicating the existence of such effects.

II. MATERIALS AND METHODS

Cultures were kept at 25°C in a constant-temperature room or at 30°C in an incubator, in both of which the relative humidity was controlled approximately within the limits 70-80 per cent. R.H. Each culture container was provided with a vial of water loosely plugged with cotton wool. Crumpled filter papers provided shelter for the animals, which were fed on a mixture of equal weights of cane-sugar and "Bemax" (a proprietary wheat germ preparation) ground up together. The food was placed in a cone of filter paper, since Woodruff and Seamans (1939) found that operated animals had difficulty in climbing out of glass food receptacles. Cultures were cleaned regularly and food, water, and shelter were renewed.

Small groups of females bearing oothecae were isolated at intervals from the mass cultures and observed daily; at each observation any newly hatched animals were counted out in as random a fashion as possible into groups of approximately 10 individuals, each such group being placed in an experimental container consisting of a muslin-covered glass cup of about 200 ml capacity, provided with shelter, food, and a water-tube as described above. From one-third to one-half of the groups from a given batch were taken at random as control material, and the rest were used for operations performed on one or more of the first few days of post-embryonic life. Single oothecae produced from 12 to 42 individuals, the usual number being about 30. Several batches treated as units for experimental purposes, however, included animals hatched on the same day from two or more oothecae.

The operation consisted of removal of the left metathoracic leg at the proximal autotomy plane, i.e. between trochanter and femur, by gentle pulling of the femur with fine forceps. This technique gives remarkably uniform results, which would probably be difficult to achieve by cutting the leg. Each operated group, with the control animals of the same batch, was then examined daily until all had moulted at least once. Operations were performed on each of the first 4 days of post-embryonic life at 30°C on several groups of different origin. A similar procedure was adopted to cover the first 6 days of post-embryonic life at 25°C. Only a few operations were performed after the sixth day at 25°C, as preliminary work had shown that for the present purpose little additional information was gained by operations very late in the instar, which had a duration in the controls of about 5 days at 30°C and 10 days at 25°C.

Blattella germanica is too active at ordinary room temperatures for easy handling and accurate observation, especially of young animals. Hence, pairs of cups, one containing operates and the other the corresponding controls, were put together into a desiccator in which they were exposed to ether vapour for a few minutes before operation or observation, or both. This method was found preferable to carbon dioxide anaesthesia for the rapid and convenient handling of large numbers of individual small containers arranged in pairs. Although Chauvin (1945) stated that ether causes very high mortalities in *Blattella germanica,* there was no indication of this in the present work. The mean duration of the first four instars of controls examined daily under ether did not differ significantly from that observed in animals in another investigation (Monro, unpublished data), which were not anaesthetized at all. The intervals between moults were similar and the etherized animals were no less viable than the others.

At 25°C, but not at 30°C, however, certain hatchings, whether examined under ether or not, showed considerable mortality in both operates and controls. In a given hatching, such mortality seldom appeared random, but usually affected all or most animals in a particular container. Death was often preceded by a characteristic "dwarfish" appearance, usually recognizable within a day or two of hatching, if not at the time of hatching; this might persist for weeks before death occurred, but no moulting took place. A few recoveries were observed, but the affected animals were so delayed and erratic in their first ecdysis as to be useless for the purpose of the present work. Hence it took a considerable time to assemble a sufficient body of reliable data for animals at 25°C in which none of the operated or control groups showed signs of "dwarfism," which may be due to some form of infection affecting growth and metabolism.* The high death rate affecting both operated and control animals, occurring in some but not all of their hatchings, reported by Woodruff and Seamans (1939), may perhaps have been due to a similar phenomenon.

III. Experimental Results

(*a*) *Morphological Appearance of Regenerating Legs at the First and Second Ecdyses*

Although the operation performed involved detachment of the leg at a natural "plane of weakness" across the articulation between the trochanter and femur, this articulation is traversed by a muscle attached distally in the femur and proximally to the median wall of the trochanter (Plate 1, Fig. 1). Detachment of the femur involves tearing out this muscle. The muscle always breaks free at its proximal end, and this presumably occurs also in the frequent instances of loss of a leg without experimental interference.† The amount of damage produced in the trochanter by the operation seems to be quite uniform, although a somewhat variable amount of bleeding seems to occur before the initial closure of the wound by clotted blood. The missing region of the leg is regenerated within the coxa, and when fully developed the regenerated tarsus extends into the empty space within the trochanter, coming into contact with the blood clot (Plate 1, Fig. 2).

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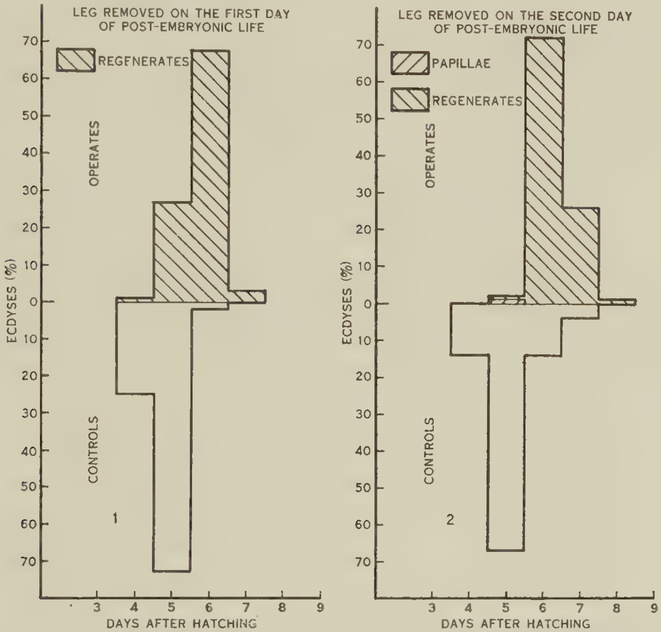
* We are indebted to Professor O. W. Tiegs, F.R.S., for drawing our attention to the presence of a microsporidian infection in the fat-body in material from our mass cultures.

† Woodruff (1937) referred to "autospasy" in *B. germanica.* This term was used by Wood (1926) for the casting of a limb when pulled by some outside agent, as distinct from the reflex shedding of the appendage implied in the term "autotomy." Our observations leave us uncertain which term is strictly applicable to the mechanism operating in *B. germanica,* and the more familiar term "autotomy" is therefore retained.

When ecdysis occurs within a few hours after operation at 30°C, the blood-clotted trochanter persists more or less unchanged in outward appearance through the next instar, and a fully differentiated regenerate appears at the second moult after the operation. Lapse of a longer period between operation and ecdysis permits a varying amount of closure of the wound by newly formed cuticle, apparently at the expense of some resorption of tissue, so that animals moulting more than 24 hr after operation have the trochanter replaced by a more or less well-rounded papilla, typically smaller than the normal trochanter, with little or no trace of clotted blood (Plate 1, Fig. 3). This condition persists through the ensuing instar, and at the second ecdysis after operation a fully differentiated regenerate appears. Such papillae occur at 30°C when the interval between the operation and the next ecdysis does not exceed 3 days (exceptionally 4 or 5 days), and at 25°C when this interval does not exceed 6 (exceptionally 7-9) days. At both temperatures, the papillae were observed to vary considerably in size, and slightly in general shape and in the number and arrangement of setae present. These variations were not recognizably related to the timing of operation, except insofar as some of the papillae appearing an unusually long time after operation, mentioned above as exceptional occurrences, were rather larger than usual and seemed to contain a somewhat more differentiated blastema. They did not, however, resemble an intermediate stage in differentiation between the papilla and the fully formed regenerate, being simply large papillae without any external differentiation.

Fully differentiated regenerates (Plate 1, Fig. 4), consisting of femur, tibia, and four tarsomeres with a pretarsus, appear at the first ecdysis after operation when the interval between the operation and the ecdysis exceeds 4 days at 30°C, or 7-8 days at 25°C. Such regenerates are obviously smaller than the corresponding normal leg of the same animal, although in our material they were seldom less and often considerably more than two-thirds of its length. (Biometrical information on this point was provided by Woodruff and Seamens (1939), who also showed that the regenerate approximates more closely to the size of the normal leg at successive ecdyses after its first appearance.) While a detailed account of the morphology and histology of regenerates and papillae will be given in a future paper, it is necessary to mention here that the regenerates observed by us varied appreciably in morphological detail (e.g. arrangement of musculature, size and number of spines and setae) as well as in size. But the differences from the normal leg were confined to these points and to the presence of only four instead of five tarsomeres. All the gross features of the normal leg were well differentiated and clearly recognizable even in the rare instances of gross abnormality, e.g. reduplication. Nothing resembling an intermediate structure between the papilla and the fully differentiated regenerate was ever observed, and there seemed to be no relationship between the size or differentiation of regenerates and the timing of operation. These general statements apply also to regenerates appearing at the second ecdysis after operation, following production of a papilla at the first.

In short, there appears at the first ecdysis after operation *either* a papilla *or* a fully differentiated regenerate, but *never* an intermediate condition, and an "all or nothing" principle appears to be involved.



Figs. 1, 2.—Daily frequency of first ecdysis in regenerating *Blattella germanica* and in the corresponding controls, expressed as percentages of total number of operates or controls used in each series of experiments, 30°C, 70-80 per cent. R.H.

(*b*) *Effect of Regeneration on the First Post-operational Moult*

Both at 25 and 30°C, production of a regenerate at the first ecdysis after operation leads to this ecdysis being delayed, in comparison with that of the corresponding controls, by a period roughly equal to the age of the animals at the time of operation. No such delay occurs when only a papilla is produced at the first post-operational ecdysis. At both temperatures, operations on the second day of post-embryonic life or later, if they result in production of a regenerate at the next ecdysis, are separated from that ecdysis by a mean time interval slightly less than the mean duration of the first instar in the corresponding controls. This time interval is, however, distinctly greater than the duration of the control first instar for operations performed on the first day of post-embryonic life. These results are summarized for 30°C in Figures 1-4 and Table 1, and for 25°C in Table 2. The data are based on daily counts of the number of animals which had moulted at any time during the 24 hr preceding each such count. In recording the data, no distinction was made

Table 1

MOULTING OF REGENERATING ANIMALS AFTER OPERATIONS AT VARIOUS AGES IN THE FIRST INSTAR AT 30°C, 70-80 PER CENT. R.H.

Age at Operation (days)	Number of Animals Used		Numbers and Mean Ages (days) at First Ecdysis of Animals Producing Papillae and Regenerates, and of the Corresponding Controls			
	Operates	Controls	Papillae	Controls	Regenerates	Controls
0-1 (Fig. 1)	81	80	0	—	81 6·03±0·06	80 5·26±0·05
1-2 (Fig. 2)	147	135	1 (5·5?)	—	146 6·77±0·04	135 5·57±0·06
2-3 (Fig. 3)	126	120	90 5·13±0·07	120* 5·27±0·06	36 7·72±0·07	89* 5·52±0·07
3-4 (Fig. 4)	96	91	88 5·22±0·09	91* 5·27±0·10	8 8·63±0·21	56* 5·57±0·12

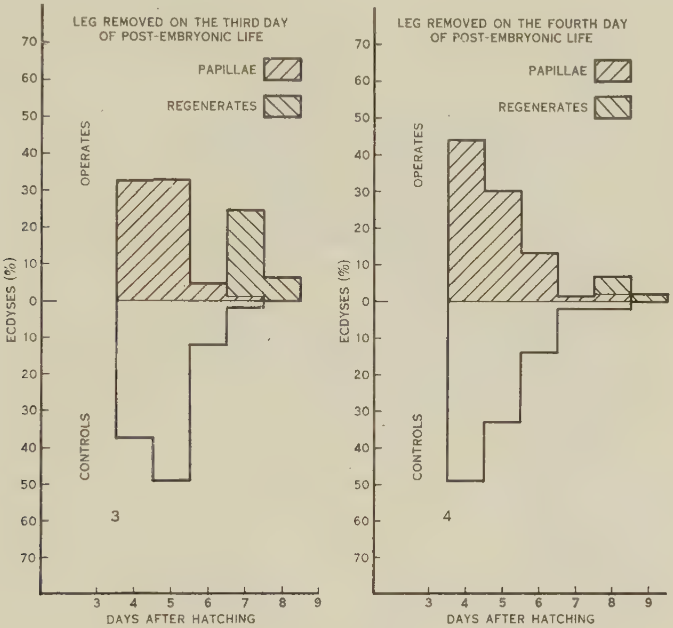
Table 2

MOULTING OF REGENERATING ANIMALS AFTER OPERATIONS AT VARIOUS AGES IN THE FIRST INSTAR AT 25°C, 70-80 PER CENT. R.H.

Age at Operation (days)	Number of Animals Used		Numbers and Mean Ages (days) at First Ecdysis of Animals Producing Papillae and Regenerates, and of the Corresponding Controls			
	Operates	Controls	Papillae	Controls	Regenerates	Controls
0-1	22	21	0	—	22 12·0±0·2	21 10·3±0·2
1-2	76	60	0	—	76 11·4±0·1	60 11·2±0·15
2-3	65	67	0	—	65 11·6±0·15	67 9·8±0·15
3-4	85	99	4 9·8±0·5	—	81 13·0±0·1	99 10·2±0·1
4-5	61	49	37 9·4±0·2	49* 9·6±0·2	24 14·7±0·2	34* 10·4±0·1
5-6	70	66	64 9·8±0·2	66* 9·6±0·1	6 15·7±0·3	34* 10·3±0·1

* These figures in Tables 1 and 2 refer to the number of control animals originating from the same hatchings as the operated animals. Since all batches of experimental animals operated at or after the "critical period" produced papillae in at least a proportion of individuals, the figure appearing for controls is equal to the total number of controls used. Regenerates, however, were produced only by a proportion of the late-operated batches, and the figures in the last column refer only to the controls of these batches, which also contribute to the total given in the fifth column of the tables.

between pale, freshly moulted individuals and those which had already hardened and darkened completely at the time of observation. Since the process of hardening and darkening occupies some hours, such a distinction would have necessarily been very arbitrary and would scarcely have improved the precision of the information obtained. The variability of the material was such that it is also doubtful whether any gain would result from shortening the interval between counts to less than the 24 hr here adopted.



Figs. 3, 4.—As for Figures 1 and 2.

Re-examination of these results shows that very few operated animals moulted on the fourth day after operation at 30°C, or on the sixth to eighth days after operation at 25°C, irrespective of the timing of the operation in relation to the duration of the instar in the controls, or of whether a regenerate or a papilla was produced. Figures 5 and 6 clarify this point, by presenting all the available data for 30°C and 25°C respectively, plotted, regardless of age at operation, with the day of operation as the zero of the time scale. Thus the "all or nothing" regeneration appears to be the result of an inhibition of ecdysis during the period in which differentiation of the regenerate from the initial blastema is taking place. A preliminary study of whole-mount preparations suggests that this period of differentiation is of fairly short duration, and the experimental data indicate that it occupies about 1 day at 30°C and 2 or 3 days at 25°C. It further appears that there is a brief "critical period," rather less than half-way through the instar. Operations performed before this period lead to appearance at the next ecdysis of a fully differentiated regenerate, the ecdysis being inhibited until differentiation of the regenerate is complete.

Operations after the "critical period" result in the appearance of a papilla at the next ecdysis, which is not delayed.

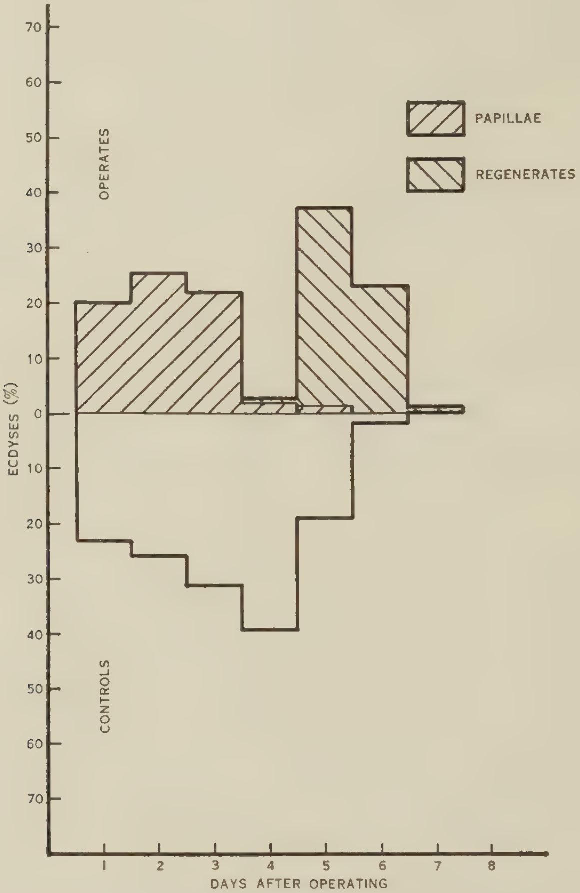


Fig. 5.—Daily percentage frequency of first ecdysis of all operated and control animals at 30°C, 70-80 per cent. R.H., plotted with day of operation as zero on the time scale.

One cannot, of course, predict by inspection whether a given batch, undergoing operation at or about the estimated time of the "critical period," will have controls moulting earlier or later than the average. Early moulting by the controls will indicate that the "critical period" was already over at the time of operation in most of the operated animals, which will therefore produce papillae. This explains the apparent association in Tables 1 and 2 between the production of papillae by operates and early moulting of the corresponding controls, which may be treated as spurious for the purpose of interpreting the present results. The most satisfactory results obtained were those in which the operation coincided with the "critical period." The natural variability of the material then resulted in a clear-cut split in the timing of ecdysis in the operated animals, some of which moulted simultaneously with the controls and produced papillae, while others moulted much later than the controls and produced fully differentiated regenerates. Such a division within a single batch of uniform origin was observed at 30°C in 10 batches and at 25°C in seven batches. The few observed instances of moulting at an abnormal time (see above) occurred mainly in these batches, as might be expected if the critical period is itself of very short duration and primarily responsible for the "all or nothing" regeneration.

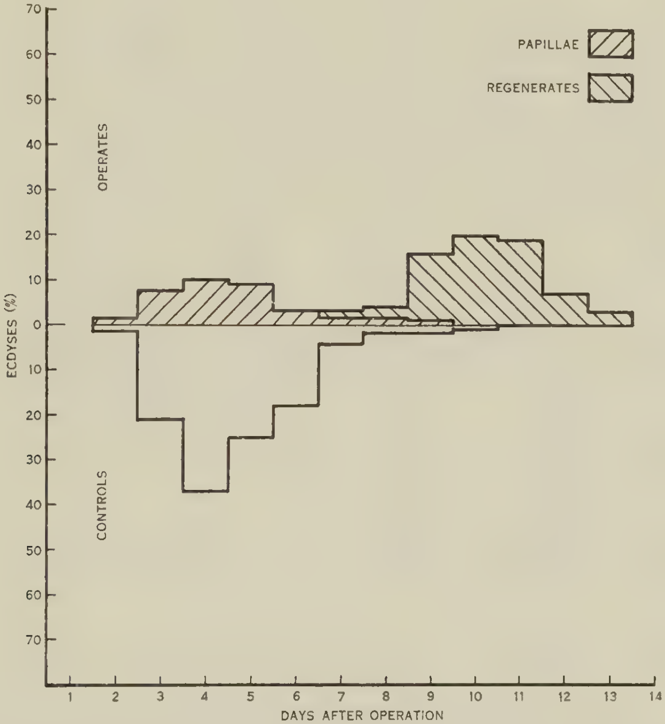


Fig. 6.—Daily percentage frequency of first ecdysis of all operated and control animals at 25°C, 70-80 per cent. R.H., plotted with day of operation as zero on the time scale.

(*c*) *Effect of Regeneration on Ecdyses After the First*

Figure 7 summarizes the data obtained from 12 batches, comprising 160 operates and 156 controls, in which operations were performed on either the first or second day of post-embryonic life, and daily observations continued until all surviving animals (118 operates and 114 controls) had completed their fourth ecdysis. All the operates produced fully differentiated regenerates at the first ecdysis, which was significantly delayed in comparison with that of the controls. The fourth ecdysis, however, although spread over a rather long period, seemed to be almost identical in timing for operates and controls.

Similar data are presented in Figure 8 for five batches (seven oothecae) including 102 operates and 103 controls, in which operations on the third and fourth days of post-embryonic life resulted in production of papillae at the first ecdysis by all but two of the operated animals. These ecdyses with papillae were simultaneous with those of the corresponding controls; regenerates appeared only at the second moult, which was slightly, but not significantly, delayed in comparison with that of the controls. The fourth ecdysis, survived by 43 operates and 43 controls only, was apparently identical in timing for both groups.

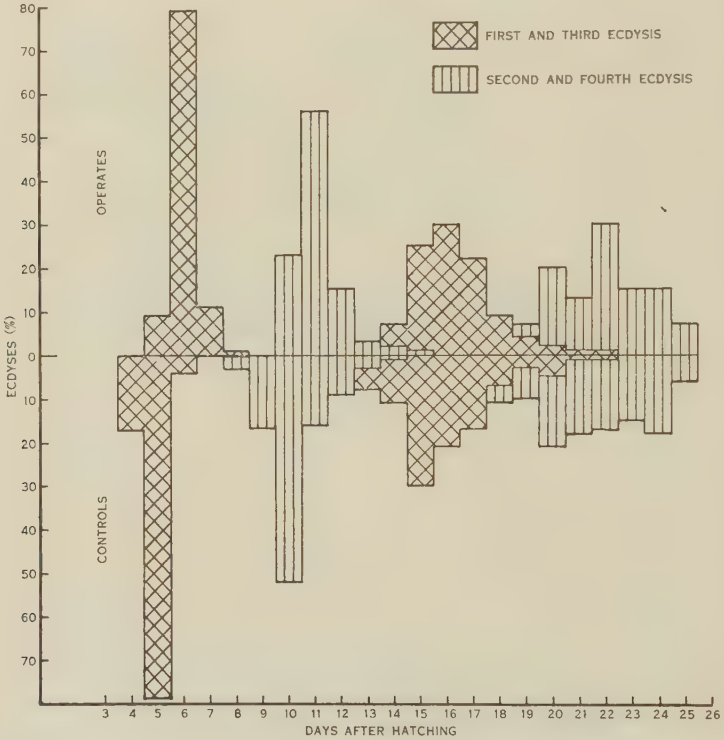


Fig. 7.—Daily percentage frequency of the first four ecdyses of operated animals producing regenerates at their first ecdysis, and of the corresponding controls. 30°C, 70-80 per cent. R.H.

Table 3 interprets these data in terms of mean duration of the first four instars. The wide spread and erratic incidence of the third and fourth ecdyses in particular make it impossible to confirm these figures statistically, since the data do not provide an adequate indication of the statistical identity of the operated and control populations at the fourth ecdysis. The experience of the authors with the rearing of *B. germanica*, together with some indications derived from preliminary work on a limited scale at 25°C, leads them to the provisional interpretation of the results in terms of a speeding up of the moulting cycle in operated animals whose ecdysis has been delayed by production of a regenerate. This would lead to the moulting of the operates "catching up" with that of the controls. Larger numbers of animals, with less erratic moulting, would be required to permit statistical evaluation of this suggestion.

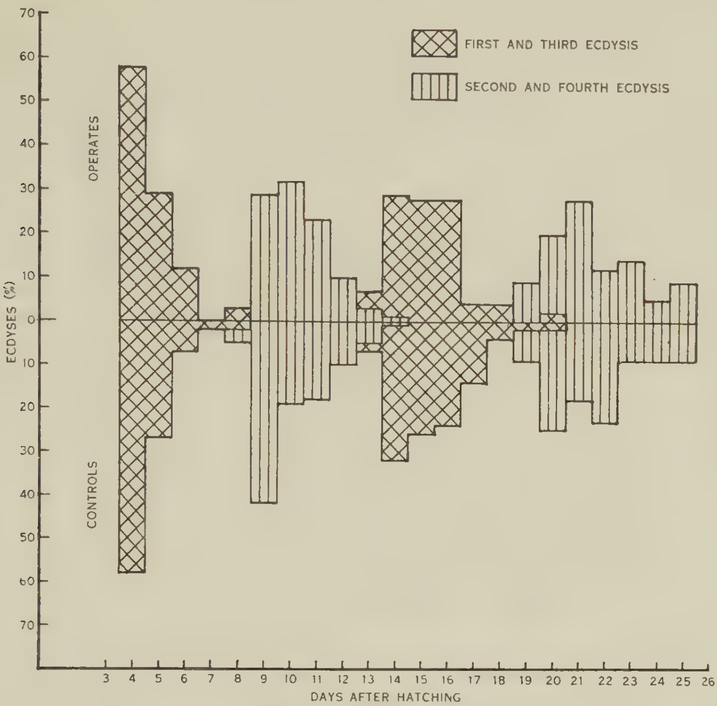


Fig. 8.—Daily percentage frequency of the first four ecdyses of operated animals producing papillae at their first ecdysis, and of the corresponding controls. 30°C, 70-80 per cent. R.H.

IV. Discussion

(*a*) *"All or Nothing" Regeneration*

In most arthropods so far studied, including insects, intermediate stages in the differentiation of regenerating appendages can be observed. In Crustacea, this often seems to occur without the intervention of a moult, the onset of differentiation being determined more or less independently of ecdysis, perhaps by re-innervation of the blastema (Needham 1945). But in insects the successive stages in differentiation usually appear at successive moults, the details of the process varying with the time of operation, and with the capacity for regeneration of the species concerned. This gradual type of regeneration is best known in *Carausius* (e.g. Heldmann 1929; Friedrich 1930), and probably occurs in many other insects, e.g. in *Rhodnius* (Luscher 1948). In such insects, an undifferentiated structure, with or without a blood clot, appears at the first post-operational moult when the operation takes place so late in the instar that the cuticle of the next instar has already begun to form, as shown in *Carausius* by Voy (1951) and in *Rhodnius* by Luscher (1948). But an "all or nothing" type of regeneration like that of *B. germanica* does not seem to have been described in arthropods, except perhaps for the observations of Cameron (1926) on the myriapod *Scutigera.*

It is worth noting that Brindley (1897, 1898), in describing regenerates obtained from operations on many individuals of *Blatta orientalis* of unknown ages, does not seem to have encountered anything that could be regarded as intermediate between the papilla and the fully differentiated regenerate as defined here. If such intermediates do not occur in *B. orientalis* it would dispose of the suggestion that the peculiarities of regeneration in *Blattella germanica* are due to its high growth rate and brief intermoult periods under the conditions of our experiments; for *B. orientalis* grows far more slowly and has far longer intermoult periods than *B. germanica* (Laing 1946).

TABLE 3

DURATIONS OF FIRST FOUR INSTARS IN OPERATED AND CONTROL ANIMALS AT 30°C, 70-80 PER CENT. R.H.

		Mean Duration of Instar (days)				Mean Age at Fourth Ecdysis (days)
		I	II	III	IV	
Operated animals	Producing regenerates at first ecdysis	6·5	5·1	5·2	5·8	22·6
	Producing papillae at first ecdysis	5·1	5·6	5·0	6·4	22·1
All controls		5·25	5·4	5·6	6·05	22·3

It is possible, however, that the "all or nothing" effect results from the ability to produce a fully differentiated regenerate in something less than the duration of an instar. Here, the processes of histolysis and histogenesis involved are likely to be much more drastic than in regenerations taking several instars to complete (irrespective of the actual lapse of time involved). If so, the cuticle of the old coxa may play a part analogous to that postulated by Hinton (1948) for the pupal cuticle of Holometabola, and the inhibition of ecdysis may arise partly from epithelial discontinuities (Wigglesworth 1950, p. 45) produced by the extreme activity of the regenerating hypodermis, especially in the formation of new muscle attachments and apodemes. But this explanation is not wholly satisfactory in view of Luscher's (1948) observation that moulting in regenerating *Rhodnius* seems to depend upon re-establishment of cuticular continuity, but appears to be independent of the stage of differentiation attained by the regenerate. Moreover, the results here described suggest that production of a fully differentiated regenerate at the first ecdysis after operation subjects the whole moulting process to a fresh start, the ecdysis being delayed by a period roughly equal to the age of the animal at the time of operation. There is also some evidence that subsequent ecdyses are affected. No such effects are associated with the production of a papilla at the first post-operational ecdysis, whether the cuticular continuity over the papilla has been re-established or not. Finally, the regular and predictable relationship of papillae and regenerates to the "critical period," in contrast to the fact that their variation in size and structure is unrelated to the timing of the operation, render an interpretation solely in terms of temporary epithelial discontinuity during regeneration rather unsatisfactory, and it seems desirable to consider possible relationships between regeneration and the endocrine balance controlling moulting.

(*b*) *Regeneration and the Endocrine Balance Involved in Moulting*

Current theories on the control of moulting and metamorphosis in insects (e.g. Scharrer 1946, 1948, 1952; Wigglesworth 1948*a*, 1948*b*, 1950, 1952*a*, 1952*b*; Novak 1951*a*, 1951*b*; Williams 1952) postulate the operation of an interacting hormone system under the control of the brain. Major roles are played by the "juvenile" hormone from the corpora allata, and by the "moulting" or "growth and differentiation" hormone. The latter originates from thoracic glands, under the influence of the brain, in Lepidoptera (Williams 1947), in *Rhodnius* (Wigglesworth 1952*a*), and in the cockroach *Periplaneta* (Bodenstein, quoted by Wigglesworth 1952*a*). Thoracic glands are present in the cockroach *Leucophaea* (Scharrer 1948) and have been observed in *Blattella germanica* in our laboratory (Wickham, unpublished data 1952) but their function has not been demonstrated experimentally.

Much of the experimental work on which these theories are based involves "all or nothing" responses, e.g. in extirpation of the corpora allata in *Leucophaea* (Scharrer 1946). The corpora allata are necessary to successful differentiation of regenerates in *Carausius* (Pflugfelder 1939). It therefore seems reasonable to assume that the "critical period" here described for regeneration in *B. germanica* bears some relation to the critical period found in experimental studies of the functions of the corpora allata and thoracic glands. The secretory cycles of these organs are usually minimal about the time of ecdysis, and reach their peak somewhere about the time in the instar at which the changes in the hypodermis leading to formation of a new cuticle are initiated. The presence of both hormones (or hormone complexes) in adequate concentration seems to be necessary for moulting to take place (e.g. Scharrer 1946). If regeneration imposes a drain on the supply of one or both hormones, the following theoretical picture results:

Operations late in the instar, after the critical period, initiate regeneration when the demand of the tissues in general for hormones has been met, the changes leading to ecdysis have been irreversibly established, and the secretory cycle of the endocrine organs is declining towards its minimum. No delay in ecdysis is likely under these conditions, and the low level of hormones in the blood cannot provide for extensive differentiation. Production of a papilla, without delay in ecdysis, is therefore to be expected. With operations before the critical period, however, regeneration, with the "priority" over normal growth often postulated by Przibram (e.g. 1919), competes successfully for hormone supplies with tissues whose response has not yet been determined. The competence of the tissues in general to respond to the moulting hormone is thus reduced, and is not restored until the hormone demands of the differentiating regenerate have been met; rapid differentiation, as seen in *B. germanica,* is likely to impose a much more severe drain on hormones, so that tissue competence is not merely reduced but abolished, and no ecdysis occurs until the normal endocrine balance has been restored. Wigglesworth (1952*b*) has observed that in *Rhodnius* the maximum secretion of juvenile hormone probably occurs after the critical period, which coincides rather with the secretory peak of the thoracic glands. The postponement of ecdysis in regenerating *B. germanica* by a period equal to the age of the animal at operation, however, suggests that the hormone demands of the regenerate have a more profound effect on the overall endocrine balance with operations near the critical period than with those performed early in the instar. This leads to the suggestion that the secretory cycle disturbed must be one which has its peak about the critical period, rather than after it, or else that the critical period for regeneration is in fact slightly different from the critical period for the endocrine system. So far as it goes, the above theoretical picture is compatible with the results described in the present paper for the effects of regeneration on the first post-operational moult, and conforms with the view that the non-appearance of intermediate stages in differentiation of the regenerate is due to an inhibitory influence of differentiation on the moulting process.

Other findings here described also conform with such an interpretation. Operations on the first day of post-embryonic life lead to an unexpectedly prolonged delay in ecdysis, but this is not surprising if it is assumed that a disturbance of endocrine balance is involved. Such a disturbance, beginning at or before the initiation of secretory activity in the endocrine organs, might well have a greater effect than one initiated when the secretory cycle was well under way, but short of its peak. The similarity of the results for all animals undergoing operation on the first day, whether still pale and soft or fully hardened and darkened, renders this explanation at least as satisfactory as the suggestions that the apparent anomaly may be due either to a specially severe "operational shock" in very young animals, or to their failure to ingest any food before operation. Again, the evidence here presented for a speeding up of the moulting cycle in later instars to compensate the delay in the first ecdysis is not incompatible with an interpretation in terms of hormone effects. If the delayed ecdysis is due to abolition of tissue competence towards the moulting hormone, it is possible that an excess of this hormone may accumulate in the blood, and Williams (1952) has shown that the moulting hormone in lepidopterous pupae may activate the prothoracic glands to produce further supplies. Hence there is a theoretical possibility that the rate of production of moulting hormone in instars following a delayed ecdysis in *B. germanica* may be increased, its threshold concentration may be reached abnormally early in the instar, and one or more accelerated ecdyses may result.

It is of course impossible to exclude the hypothesis that some of the effects observed result from preferential utilization by the differentiating regenerate of food materials and metabolites other than hormones. The general conformity of the results with a theoretical picture based on current views of hormone action, however, seems fairly satisfactory. There is as yet no confirmation of the belief induced by the results of Pflugfelder (1939) that the hormone mainly concerned is the juvenile hormone, which would tend to support the view expressed by Novak (1951*b*) that the moulting hormone has little, if any, direct effect on growth or differentiation. The possibility of using regenerating *B. germanica* in studies on moulting and metamorphosis, as well as on regeneration itself, seems to be sufficiently established to justify further experimental and histological work on all three aspects, which it is hoped to publish in future papers of this series.

V. Acknowledgments

We are indebted to the Research Committee of the New England University College for providing funds to assist this work; to Mr. J. M. Munro and Miss C. A. Wickham, of this laboratory, for permission to quote unpublished observations; and to Professor P. D. F. Murray, Dr. John A. Moore, and Dr. M. F. Day for reading the manuscript and offering valuable suggestions and criticisms.

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EXPLANATION OF PLATE 1

Blattella germanica L.

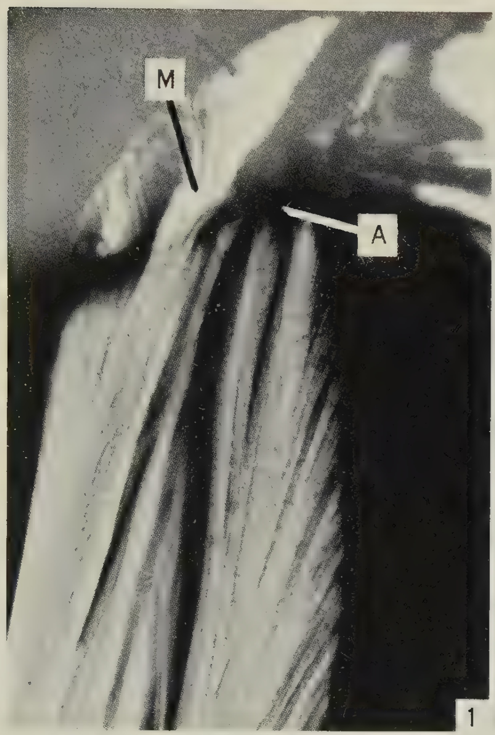
Fig. 1.—Trochanter and adjacent regions of the normal metathoracic leg in the first instar, showing the plane of autotomy at the trochantero-femoral articulation (*A*), traversed by a muscle (*M*). Whole mount stained with eosin and photographed by polarized light.

Fig. 2.—Coxa and trochanter of an operated metathoracic leg, photographed just before the first post-operational ecdysis, showing the dark blood clot (*B*) closing off the old trochanter, and the newly formed regenerate folded within the stump of the old leg.

Fig. 3.—Bases of the normal and operated metathoracic legs of an animal, operated on the third day of post-embryonic life at 30°C, after its first post-operational moult at 5 days old, showing a typical smoothly rounded papilla on the operated side.

Fig. 4.—Regenerated and normal metathoracic legs of an animal, operated on the day of hatching at 30°C, after its first post-operational moult at 6 days old. Note the completeness of differentiation of the regenerate, which differs from the normal leg mainly in size and in the presence of only four tarsomeres.

REGENERATION AND MOULTING IN *BLATTELLA*. I



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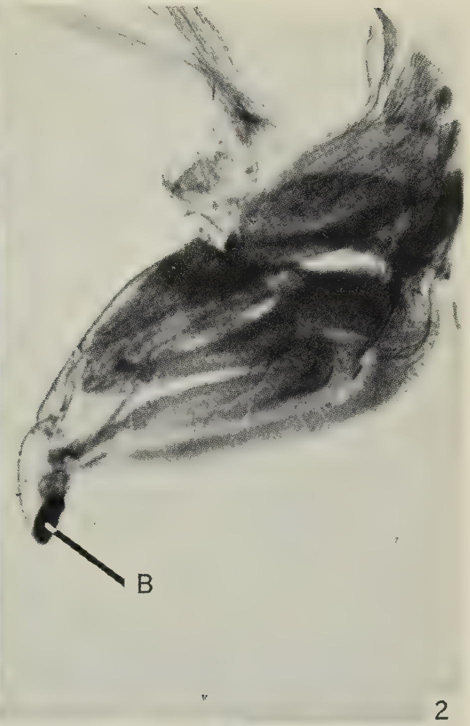

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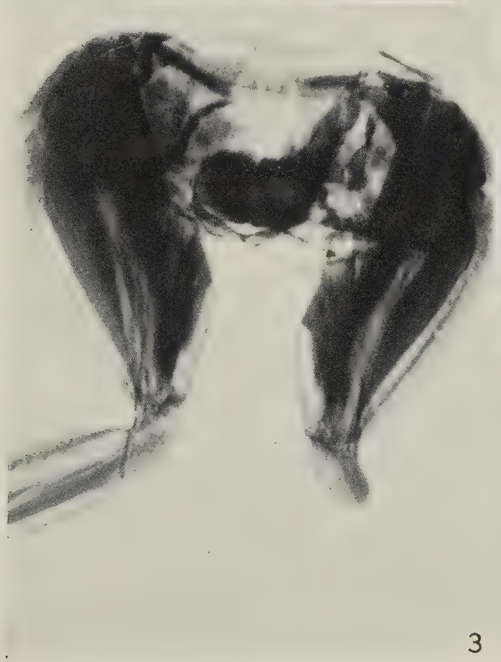

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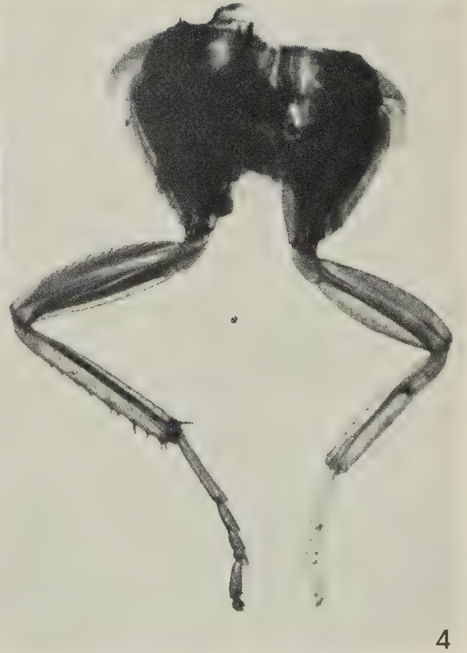

4

THE EFFECT OF BORATE ON THE OXYGEN UPTAKE OF BRAIN TISSUE IN KREBS PHOSPHATE RINGER SOLUTION

By E. M. TRAUTNER[*] and M. MESSER[*]

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Summary

The effect of borate on the respiration of guinea pig brain cells in Krebs phosphate Ringer solution in the absence of any added substrate and in the presence of glucose and fructose has been investigated.

In the absence of substrate borate rapidly depresses the oxygen uptake, causing finally a very low, apparently constant "borate-resistant" respiration.

In the presence of glucose in concentrations sufficient to maintain the respiration rate of the tissue in the absence of borate, the addition of borate in 50 to 100 times excess over the glucose has no early effect on the respiration.

To inhibit the effects of borate to the same degree, fructose has to be present in four to six times higher concentration than glucose.

In the absence of additional hexose, the R.Q. of the tissue respiring in the presence of borate falls rapidly below 0.9. In the presence of a sufficient concentration of the two sugars it remains unaltered, slightly lower than 1.0.

Addition of 0.03M mannitol to the medium has no effect on the action of borate.

Glucose in very low concentrations maintains the normal respiration rate of the tissue in the presence of borate for a period depending on its concentration. Increasing concentrations progressively delay the onset of the respiratory depression. Fructose in low concentrations fails to maintain the respiratory rate; increasing concentrations merely raise the oxygen uptake until it coincides with that of the borate-free control.

The observations are tentatively discussed; the investigation is being continued.

I. INTRODUCTION

Cases of borate poisoning due to accidental ingestion, to prolonged lavage, or merely to extensive use of boracic powders for epidermal lesions such as eczema, have been reported repeatedly, and fatal intoxications have occurred (Abramson 1949; Rivas 1950). The drug affects particularly the central nervous system, causing convulsions, stupor, and coma; in post-mortem examinations the concentration of borate in the brain was found to be considerably higher than in the body fluids and other organs (Pfeiffer, Hallman, and Gersh 1945). It appears, however, that the effects of borate on nervous tissue have not been investigated in detail. The present work deals with the effect of borate on the oxygen uptake of guinea pig brain cells in Krebs phosphate Ringer solution. It will be shown that the drug exercises a strong inhibitory effect on the oxygen uptake of the tissue, that this effect is counteracted by glucose and fructose, and that, in the presence of borate, there are significant differences in the utilization of low concentrations of glucose and fructose.

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II. Materials and Methods

Guinea pigs were killed by a blow on the neck. A brain mince was obtained by pressing the cerebrum through a 60-mesh copper wire sieve. The mince consisted of thin shreds, 2-3 mm long, which on microscopic examination showed mainly intact cells and very little debris. After centrifugation of the mince in phosphate Ringer solution containing glucose, the supernatant showed no oxygen uptake; whilst the respiration of the sedimented cells remained essentially unaltered. To shorten the interval between the death of the animal and the start of manometric observation, the crude mince was mostly used without previous washing. Slices were prepared by hand with the aid of a microscope slide. After insertion into the main compartment of the Warburg flask they were teased apart with pins to prevent curling and sticking. Mince was treated in the same way to ensure homogeneous suspension. No difference in oxygen uptake was observed between slices and mince. Since the preparation of slices is relatively slow, most experiments were performed with mince; all essential observations were duplicated with slices.

Borate solutions were made by dissolving borax in distilled water and bringing to pH 6.9-7.0 with concentrated hydrochloric acid. A 0.08M borax solution made in this way is approximately isotonic. Usually 0.5 ml of a more concentrated (0.2M) solution was added to 2.5 ml of the medium, thus making the final solution slightly hypertonic with a concentration of 0.13M with respect to boric acid. Control experiments with 0.5 ml of 0.4M NaCl showed that the small increase in tonicity had no effect on the respiration of the cell.

The pH of borate solutions made in this way is increased by dilution and decreased by the addition of substances forming acidic borate complexes; the pH of each ingredient was therefore adjusted to assure a final pH of the medium within the range 7.3-7.4. Control experiments showed that variations of up to 0.2 pH units made no significant difference in the oxygen uptake. (According to Elliott and Birmingham (1949) the respiration of rat brain suspensions in phosphate Ringer is optimal between pH 7.0 and 7.6.) The pH determinations were made with a Jones' pH meter (N. L. Jones, Melbourne).

For the determination of the borate content of the tissue, slices only were used. These were dried on filter paper, weighed, and after addition of a few drops of sodium hydroxide to avoid sublimation of boron trioxide, carefully ashed on platinum. The white residue was dissolved in distilled water and the boron determined by the colorimetric quinalizarine method of Berger and Truog (1939).

Glucose and fructose solutions were made by dissolving the sugar in phosphate Ringer solution. Mannitol was dissolved in distilled water.

All reagents used were of "Analar" quality; the fructose was Extra Pure (Hoffmann-La Roche, Basel).

All concentrations are given as final molar concentrations in the medium, borate concentrations being calculated as boric acid.

Oxygen uptakes were measured in standard Warburg flasks and are expressed as μl O_2 per 100 mg fresh tissue. Between 120 and 140 mg tissue were used with flasks of 25-30 ml volume containing 3 ml fluid medium. Unless stated otherwise, the conditions were: initial pH 7.3-7.4; temperature 37.5°C; gas phase air; fluid medium Krebs phosphate Ringer solution (Krebs 1933); volume of added borate solution 0.5 ml of a molarity to give a final boric acid concentration of 0.13M; volume of added hexose or mannitol 0.5 ml; time between death of animal and start of observation 40-60 min; duration of manometric observation 5 hr. Readings were taken at least every half hour. Each graph is based on at least three separate runs.

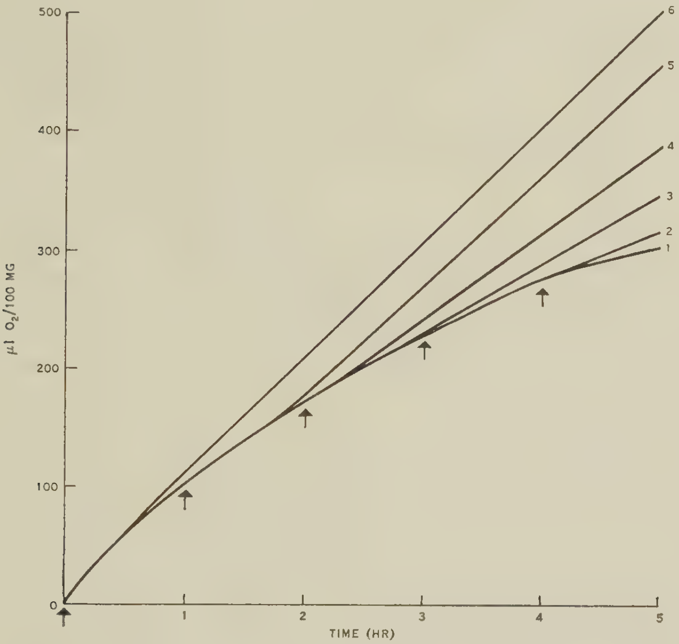


Fig. 1.—Oxygen uptake of guinea pig brain in Krebs phosphate Ringer, with glucose added after various time intervals; the ability of glucose to restore the original oxidation rate falls with increase in the time of addition. Final glucose concentration, 0.01M.

Curve 1: No glucose
Curve 2: Glucose added after 4 hr.
Curve 3: Glucose added after 3 hr.
Curve 4: Glucose added after 2 hr.
Curve 5: Glucose added after 1 hr.
Curve 6: Glucose added at start of measurement.

III. Results

(*a*) *Preliminary Observations*

(i) *Normal Respiration.*—Our preparations showed, in Krebs phosphate Ringer containing 0.01M glucose, oxygen uptakes of 90-110 µl O_2 per 100 mg fresh tissue per hour, and maintained this rate for at least 5 hr.

In the absence of glucose, the initial rate of respiration was the same or almost the same as in its presence, but very soon the oxygen uptake began to fall. There were great variations in the time of onset of this decrease, which varied from brain to brain, from 30 min to about 2 hr after start of observation. The rate of decrease reached its maximum after a further ½-1 hr; later the decrease was slower, though still steady (Figs. 1 and 4).

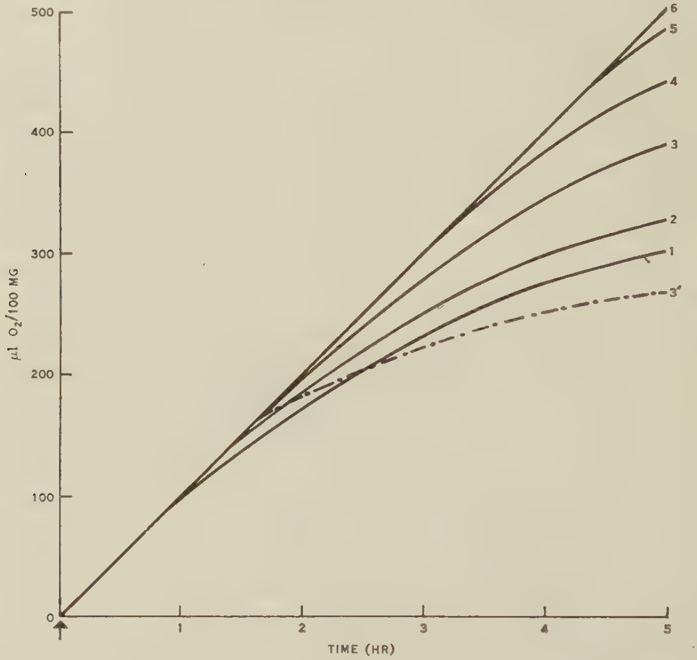


Fig. 2.—Effect of glucose at various concentrations. Glucose added at start of measurement.

Curve 1: No glucose.
Curve 2: 0.000125M glucose.
Curve 3: 0.00050M glucose.
Curve 4: 0.0010M glucose ("maintenance concentration").
Curve 5: 0.0020M glucose.
Curve 6: 0.010M glucose.
Curve 3′: 0.00050M glucose with 0.13M borate (same as Fig. 9, curve 3).

(ii) *Maintenance Concentration.*—The concentration of glucose commonly used in metabolic experiments is 0.01M; the minimum concentration capable of maintaining a constant respiration for at least 4 hr—referred to here as "maintenance concentration"—was found to be considerably lower, about 1/10 of the above concentration (Fig. 2). The maintaining effect of the glucose depended on its concentration rather than on the quantity available, since the respiration could not be maintained for a longer period by increasing the fluid volume while preserving the same low glucose concentration.

(iii) *Respiration in the Presence of Fructose.*—Under the conditions of our experiments there was no difference in the utilization of fructose and glucose. Equal concentrations of the two sugars maintained the respiration to the same degree and the maintenance concentration of both was the same.

(iv) *Late Addition of Hexose.*—When glucose or fructose (0.01M) was added to tissue which had been respiring for some time in Krebs phosphate Ringer alone, its effect depended on the degree of exhaustion of the tissue. Maintenance of the original rate of respiration was obtained only on addition during the period when the tissue still respired at its original rate or when this rate had only just begun to decline. When the hexose was added later, namely when the rate of respiration had already fallen for 20-30 min, its effect became noticeable only after a time lag (Fig. 1); the respiratory rate then increased slowly and became constant after 1-2 hr, though at a considerably lower level than that shown by the control with hexose present from the start. Very late addition of the sugars had little or no restoring effect, but it still prevented a further decline of the oxygen uptake.

(v) *Respiration in the Presence of Mannitol.*—Mannitol is one of many hydroxy compounds forming strong complexes with boric acid (cf. glycerol, α-hydroxy acids, salicylic acid). It is not toxic in the concentrations used. Its addition to brain cells in concentrations up to 0.03M was found to have no effect on the oxygen uptake of the tissue respiring either alone or in the presence of glucose or fructose.

(vi) *Relative Ability of Glucose and Fructose to Form Borate Complexes.*—It is known that "ketoses form much greater quantities of the boric acid complex than do aldoses" (Boeseken 1949); an attempt was made to estimate approximately the relative ability of glucose and fructose to form these complexes at concentrations similar to those of our experiments.

At room temperature, 20 ml of 0.1M borate solution of pH 7.4 showed, on addition of solid glucose to 0.01M concentration, a pH drop of 0.2 units and required 0.3 ml of 0.1N sodium hydroxide solution to restore the pH to 7.4. With fructose, the pH dropped by almost one unit and the solution required 1.4 ml sodium hydroxide to restore the pH, indicating that fructose forms—under these conditions—acidic complexes to an extent of about 70 per cent. of its amount, which is 4-5 times higher than with glucose. Under the actual experimental conditions (phosphate Ringer 0.13M borate, pH 7.4, 37.5°C), the extent of complex formation can be expected to be still higher.

Unfortunately it was not possible to estimate to what extent free sugars and their modifications may still be present under the conditions of our experiments. The physicochemical data available from the literature (Isbell *et al.* 1948; Boeseken 1949; Ross and Catotti 1949) shed little light on this question since they hold only under conditions vastly different from ours.

(vii) *Borate Content of the Tissue.*—Slices were incubated in borate-free and in borate-containing medium for periods between 20 and 120 min. They were then removed, rinsed with phosphate Ringer, dried, weighed, and analysed. In slices incubated in borate-free medium, no boron could be detected by the colorimetric method used. The borate content of the slices incubated in borate-containing medium was found to be, weight for weight, identical with that of the medium. The presence or absence of glucose, fructose, or mannitol made no difference to the boron content of the slices. No selective absorption of boron could thus be detected.

(*b*) *Effect of Borate Alone*

(i) *Effect on Respiration.*—When brain slices or mince, directly after preparation, were suspended in a medium containing 0.05-0.15M boric acid, their respiration, compared with that of the controls, had decreased considerably by the time manometric measurement could be started. The degree of this depression depended on the concentration of borate rather than on its quantity.

Borate concentrations of the order of 0.001M and lower had no effect on the oxygen uptakes during the experimental time.

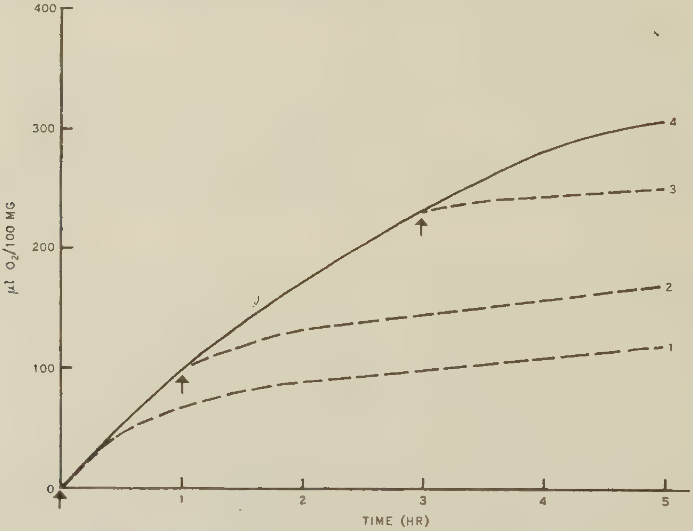


Fig. 3.—Borate added after various time intervals; no glucose. Time-lag on early addition. Borate concentration 0.13M (expressed as H_3BO_3).

Curve 1: Borate added at start of measurement.
Curve 2: Borate added after 1 hr.
Curve 3: Borate added after 3 hr.
Curve 4: No borate.

(ii) *Onset of the Borate Effect.*—When borate was added to the medium at the start of manometric observation, the onset of the respiratory depression became noticeable only after a delay (Fig. 3), which varied from brain to brain from 15 min to almost 1 hr. The effect appeared to be more delayed if the borate-free control was able to maintain a constant respiration rate for 1-2 hr, but to occur earlier if the tissue alone was unable to maintain its original respiration. Once borate had begun to show its effect, the rate of oxygen uptake decreased rapidly, a maximum rate of decrease being reached after about 30 min and maintained for a short time. Later the decrease became slower, and finally an almost constant very low rate of oxygen uptake was reached and maintained for the rest of the experimental period. This rate, 10-15 μl O_2/100 mg tissue per hour, was lower than the final rate reached by the borate-free controls within the same time, and it appeared to be almost equal with all brains examined. The nature of this "borate-resistant" oxidation was not further investigated.

When the borate was added at a time when the tissue showed already signs of exhaustion insofar as its respiration had begun to decline, the initial period of delay was no longer pronounced. The oxygen uptake dropped almost immediately to the final low value, which was the same, no matter at which stage the borate had been added (Figs. 3 and 4).

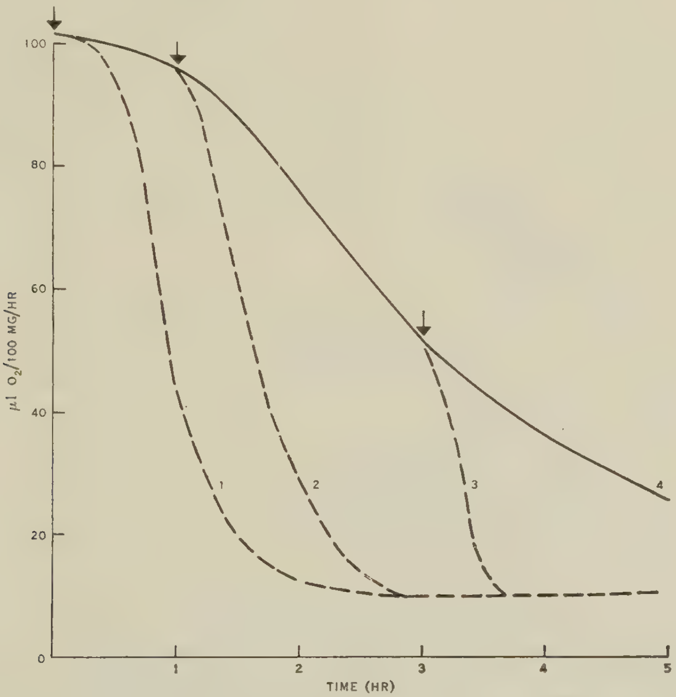


Fig. 4.—Rate of change in oxygen uptake for the experiment illustrated in Figure 3. Identical low, final rate in the presence of borate. Curves numbered as in Figure 3.

With lower concentrations of borate the delay in the onset of borate action was increased (Fig. 5). Under these conditions the final low oxidation rate was not always reached during the observation time. The lowest borate concentration at which this final rate was reached within 4 hr was found to be of the order of 0.05M.

(iii) *Reversibility of Borate Action.*—Fresh brain slices were incubated in borate-free and in borate-containing medium, removed after various time intervals, rinsed with Krebs-phosphate Ringer solution, and brought to manometric observation in Krebs-phosphate Ringer containing 0.01M glucose. Slices which had been under the influence of borate for over 15 min. showed initially a lower respiration rate than the controls. After 20-30 min, however, their oxygen uptake increased slowly, to reach after 2-5 hr that of the control (Fig. 6). Partial recovery took place even when the tissue had been exposed to the action of borate for as long as 1 hr.

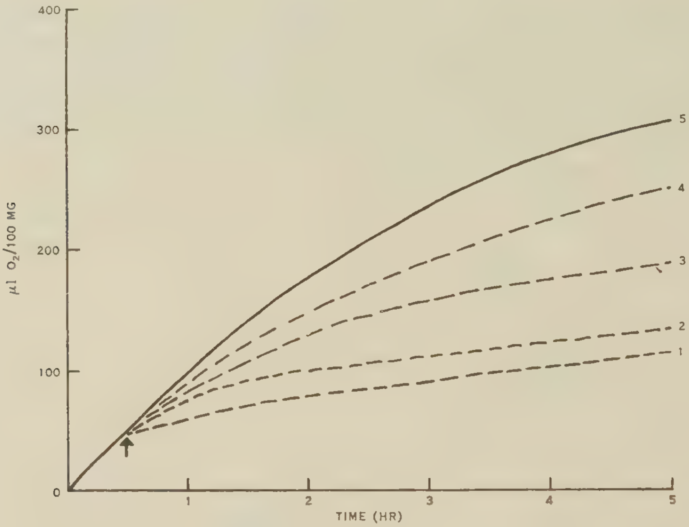


Fig. 5.—Effect of borate at various concentrations; no added substrate.

Curve 1: 0.26M borate.
Curve 2: 0.13M borate.
Curve 3: 0.065M borate.
Curve 4: 0.033M borate.
Curve 5: No borate.

(*c*) *Borate and Glucose*

(i) *Inhibition of Borate Effect.*—When borate was added to tissue already respiring in the presence of glucose sufficient to maintain its normal respiratory rate (above 0.002M), no early effects of borate were observed, no matter when the borate was added. In most cases the tissue continued to respire at its normal rate throughout the period of observation, but often a distinct, if slight, fall in the oxygen uptake was observed after 3-4 hr (Fig. 7, curve 3). This late effect of borate was noticed at low as well as at high glucose concentrations and could not be prevented by increasing the glucose concentration to 0.02M. Glucose in proportion of 1 part to up to 100 parts of borate prevented the early onset of depression by 0.13M borate.

With later addition of glucose of the same concentrations to tissue already respiring in the presence of borate, the effect of the sugar decreased with the delay in addition. Only during the first 15-20 min of borate action could glucose prevent the onset of respiratory inhibition, and only during this period was the oxygen uptake the same, no matter whether borate or glucose was added first. On later addition the effect of glucose decreased rapidly, though it still caused an increased oxygen uptake and a constant oxidation rate (Fig. 7). There was only a slight delay in the onset of the glucose effect, and no late recovery was observed (cf. Fig. 6, representing an experiment in which the tissue was removed from the borate-containing into a borate-free glucose-containing medium). Even when the tissue was already respiring at the final low rate caused by borate, there was on addition of glucose still a small but distinct increase in the oxygen uptake, although usually not sufficient to double the prevailing rate of about 10 μl O_2/100 mg/hr.

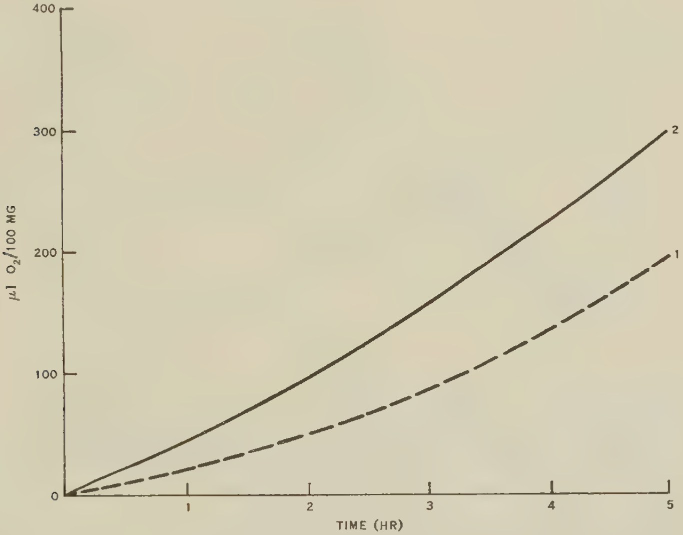


Fig. 6.—Recovery of brain slices in glucose phosphate Ringer after previous exposure to 0.13M borate in glucose-free medium. Glucose 0.01M.

Curve 1: Slices previously exposed to borate for 45 min, rinsed, and transferred into fresh medium.

Curve 2: Slices treated in the same manner without exposure to borate (control).

(ii) *Effect of Exhaustion.*—In parallel experiments tissue preparations were allowed to respire in substrate-free medium until a decline in the rate of respiration was distinctly observed. Then glucose (0.01M) was added to one flask, followed after 15 min by borate (0.13M); to the other flask the borate was added first, followed after the same time by glucose.

Although the media in both flasks were of the same final composition, the final respiration rates were different (Fig. 8). The tissue to which glucose had been added first restored and maintained the original respiration rate, while the tissue to which borate had been added first respired at a considerably lower, though also constant rate. Once the tissue became exhausted, the onset of the borate-caused respiratory depression could be prevented only by almost simultaneous addition of glucose.

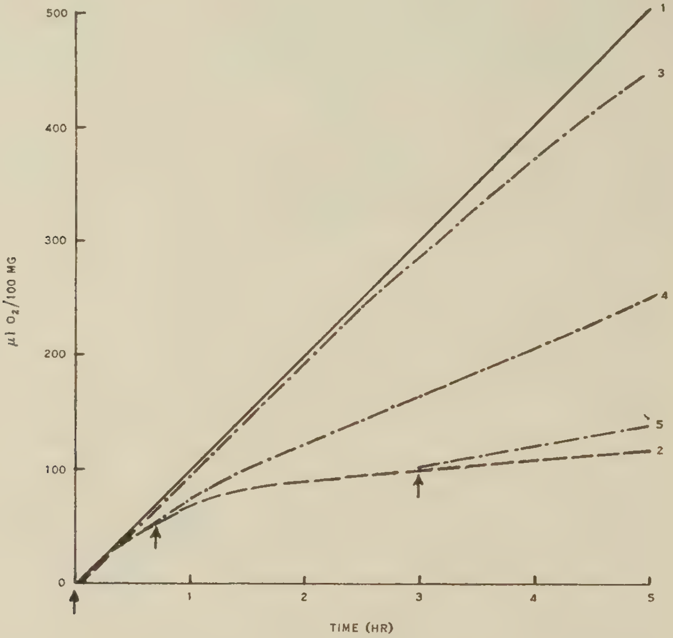


Fig. 7.—Effect of glucose added after various time intervals to brain cells respiring in borate from the start of measurement. The ability of glucose to abolish the effect of borate decreases rapidly with increase in the time of addition. Borate 0.13M; glucose 0.01M.

Curve 1: Respiration in glucose; no borate.
Curve 2: Respiration in borate; no glucose.
Curve 3: Glucose added simultaneously with borate.
Curve 4: Glucose added after 45 min of respiration in borate.
Curve 5: Glucose added after 3 hr of respiration in borate.

(iii) *Effect of Glucose Concentrations Lower than Sufficient for Maintenance.*—When glucose to concentrations below 0.002M was added early in the experiment together with 0.13M borate, two separate effects were observed (Fig. 9; compare also Fig. 2, curve 3′). One was a delay in the onset of borate action insofar as there was a period of unchanged respiration before, almost abruptly, a progressive decline in the oxygen uptake took place. The duration of this delay increased with the concentration of glucose and shifted the onset of the respiratory depression towards the end of the observation time. No simple relation between the duration of this delay and the concentration of glucose could be observed.

The second effect concerns the rate of oxygen uptake after the onset of the respiratory depression. At very low glucose concentrations the oxygen uptake followed almost that of the borate-containing, glucose-free control and the onset of the respiratory depression showed itself as a distinct discontinuity in the curve. With increasing glucose concentrations the curves became steeper and approached finally the course of the oxygen uptake shown by the borate-free, glucose-containing control. At these higher concentrations the angle between the unchanged and the depressed rate of oxygen uptake was no longer observable as a distinct discontinuity.

Very similar curves were obtained when, in the presence of a constant low glucose concentration, the borate concentration was varied (Fig. 10). Lower borate concentrations showed a later onset of the respiratory depression and a higher rate of oxygen uptake after the discontinuity.

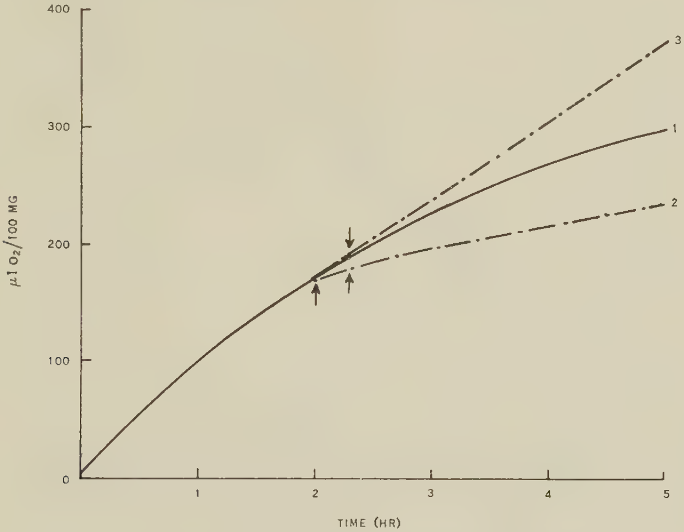


Fig. 8.—Effect of reversing the sequence of addition of glucose and borate to a preparation which had been respiring in the absence of added substrate for 2 hr. Glucose 0.01M; borate 0.13M.
Curve 1: No additions.
Curve 2: Borate added first, followed by glucose after 15 min.
Curve 3: Glucose added first, followed by borate after 15 min.

(*d*) *Borate and Fructose*

The experiments recorded in the previous section were repeated using fructose instead of glucose. The effect of 0.01-0.02M fructose was almost identical with that of the same concentration of glucose and the curves of oxygen uptakes were hardly distinguishable. It is not necessary to detail these experiments. Quantitative and qualitative differences between the two sugars were observed at lower concentrations only.

(i) *Maintenance Concentration.*—Figure 11, curves 3 and 4, represents the oxygen uptake of 100 mg tissue in the presence of 0.0025M glucose and fructose, slightly higher than the maintenance concentration. The addition of borate (0.13M) to the tissue with glucose (curve 2) had little effect on the respiratory rate, while its addition to that with fructose (curve 1) reduced the oxygen uptake by over 50 per cent. over a period of 4 hr. Invariably fructose had to be present in several times its maintenance concentration, usually between 4 and 6 times, but up to 10 times, to counteract the early effect of borate. One part of fructose thus counteracted the effect of only 10-25 times excess of borate.

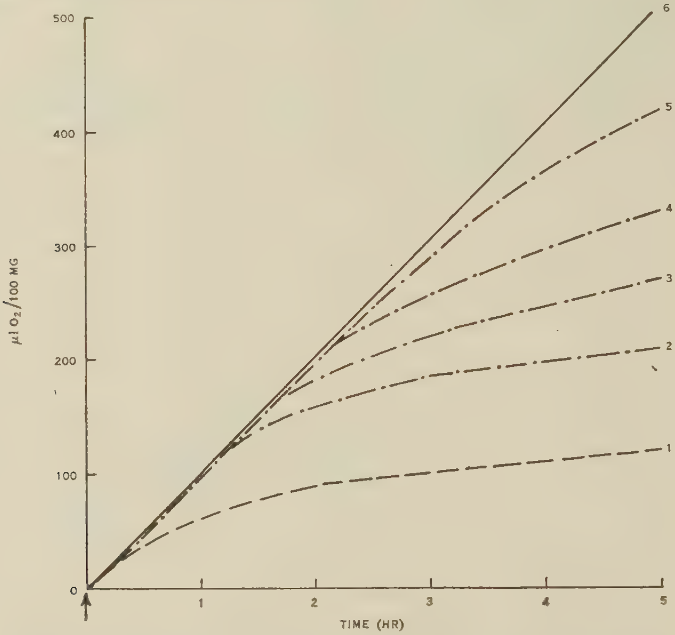


Fig. 9.—Effect of glucose in low concentrations on the respiration in the presence of 0.13M borate (cf. Fig. 2). Borate and glucose added simultaneously at start of measurement.

Curve 1: Borate alone; no glucose.
Curve 2: 0.00025M glucose.
Curve 3: 0.00050M glucose.
Curve 4: 0.0010M glucose.
Curve 5: 0.0020M glucose.
Curve 6: 0.0020M glucose; no borate.

(ii) *Low Concentrations of Fructose.*—Figure 12 records an experiment in which the same brain preparation was used as in the corresponding experiment with glucose (Fig. 9). It is seen not only that in the presence of borate the effective fructose concentrations were higher than those of glucose, but also that the oxygen uptake with fructose declined directly from the moment of addition. In no case was a delayed onset of borate action observed, nor were there any discontinuities in the curves, which were very similar to those obtained with lower concentrations of glucose *after* the onset of the respiratory depression. The same difference was observed when the borate concentration was varied in the presence of a constant, low concentration of fructose (Fig. 13; cf. Fig. 10).

(iii) *Low Fructose with Very Low Glucose Concentrations.*—It was considered that the presence of small glucose concentrations might facilitate the utilization of fructose in the presence of borate. No such effect could be demonstrated.

(iv) *Late Addition of Glucose and Fructose.*—When equal, low concentrations of the two sugars, 0.005M, were added to tissue respiring in the presence of borate, the difference between the effect of the two sugars decreased with increase in the time of borate action. At a time when the final low rate of oxygen uptake had become established, there appeared to be no longer any difference between the two sugars; but the increases in the rate of oxidation caused by very late addition were too slight to allow conclusive interpretation.

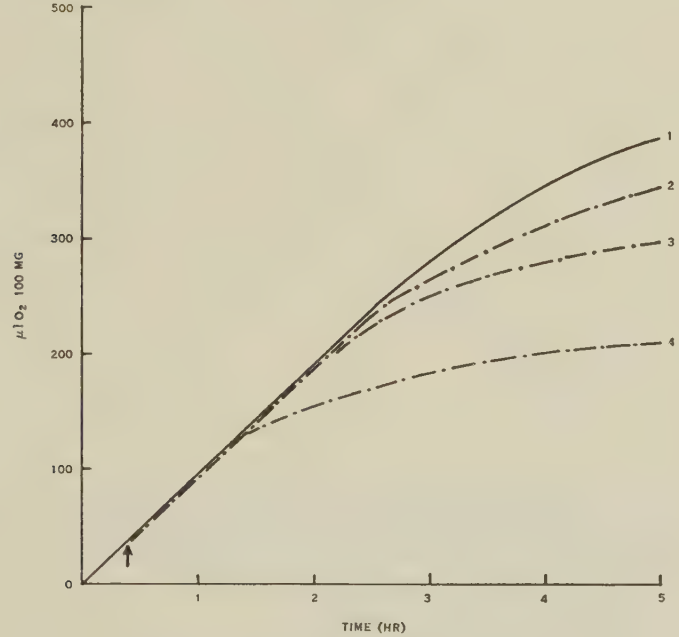


Fig. 10.—Effect of increasing concentration of borate on the respiration in the presence of a low concentration of glucose, 0.00050M. Borate and glucose added simultaneously after 25 min.
Curve 1: Glucose alone; no borate.
Curve 2: 0.065M borate.
Curve 3: 0.13M borate.
Curve 4: 0.26M borate.

(*e*) *Mannitol and Borate*

It seemed of interest to find to what extent the presence in the medium of fairly high concentrations of a strong, metabolically inert complex-former might reduce the effect of borate.

Mannitol to concentrations up to 0.03M was added to 0.13M borate and the pH readjusted after the mixture had been allowed to stand overnight. Since one molecule of mannitol probably combines with two of borate (Isbell *et al.* 1948) the amount of free boric acid in a solution of 0.13M concentration could be expected to be reduced by almost 50 per cent. by 0.03M mannitol. However, within this range of concentrations, no significant influence on the reduction of oxygen uptake caused by borate could be detected, either in the presence or the absence of low concentrations of glucose or fructose.

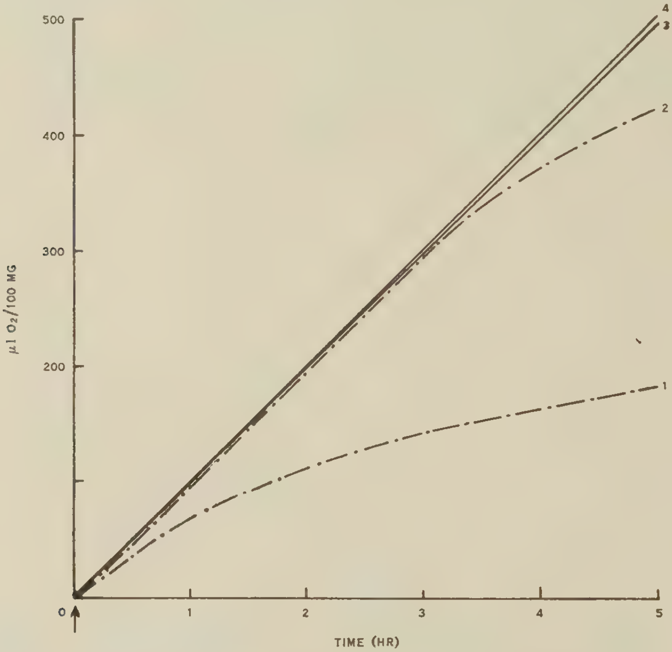


Fig. 11.—Effect of equal, low concentrations of glucose and fructose on the respiration in the presence and in the absence of 0.13M borate. Sugar and borate added simultaneously at start of measurement. Sugar concentration 0.0025M.

Curve 1: Fructose and borate.
Curve 2: Glucose and borate.
Curve 3: Fructose; no borate.
Curve 4: Glucose; no borate.

(*f*) *Respiratory Quotients*

Respiratory quotients (R.Q.) were measured in a series of experiments using the direct method of Warburg. Tissue respiring in the absence of any additional substrate showed during the first 2 hr R.Q.'s of about 0.99, later the value dropped below 0.95. (At the same time the pH in an unbuffered or only weakly buffered medium increased to above 7.5 and free ammonia could be determined, indicating that amino compounds were utilized as the exhaustion of the cell proceeded.) In the presence of borate and absence of additional substrate, the R.Q. dropped even more quickly, reaching values below 0.89 within 3 hr. These observations will be more thoroughly followed in connection with experiments on the utilization of a series of other metabolites in the presence of borate. For the present purpose, the only pertinent fact is that glucose prevents the sharp drop in R.Q. caused by borate alone. In the presence of a maintenance concentration of glucose, borate affected the R.Q. but slightly, values between 0.95 and 0.98 having been observed. No difference in R.Q. could be noted between glucose and fructose in the presence of borate as long as the concentrations of the sugars were sufficient to maintain the normal rate of respiration.

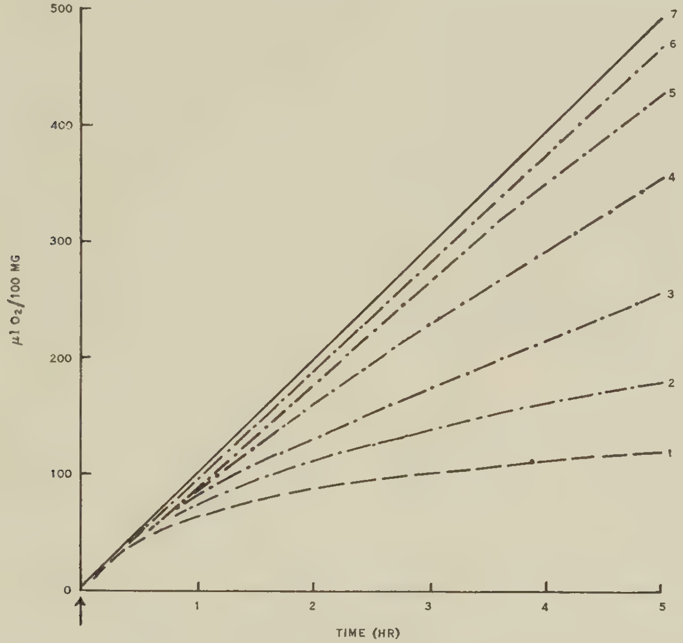


Fig. 12.—Effect of increasing concentrations of fructose on the respiration in the presence of 0.13M borate. Fructose concentrations are above the maintenance concentration. Same brain preparation as in the corresponding experiment with glucose, Figure 9. Borate and fructose added simultaneously at start of measurement.

Curve 1: Borate alone; no fructose.
Curve 2: 0.0025M fructose.
Curve 3: 0.0050M fructose.
Curve 4: 0.0075M fructose.
Curve 5: 0.010M fructose.
Curve 6: 0.020M fructose.
Curve 7: 0.020M fructose; no borate.

IV. Discussion

The experimental data presented show three points of interest: (i) in substrate-free medium borate depressed strongly the oxygen consumption of the surviving brain cell; (ii) the normal respiratory rate was, however, preserved if either glucose or fructose in concentrations of the order of 0.01M were present in the medium, but (iii) at lower hexose concentrations there was a pronounced difference between the effect of the two sugars in the presence of borate.

(i) Pfeiffer, Hallman, and Gersh (1945) observed that, on slow peritoneal irrrigation with 5 per cent. boric acid solution over 90 per cent. of the boric acid was absorbed during the first hour of irrigation, while after a period of 3 hr analysis of the brain, liver, and fat of the animals showed high borate concentrations. The drug freely penetrated the peritoneum and passed rapidly into the circulation and equally rapidly into distant tissues. Our observations were in accord with these findings insofar as already after 20 min of suspension in borate-containing medium the borate concentration in brain slices was equal to that of the medium. On early transfer of the tissue into borate-free medium, the normal rate of oxygen uptake was slowly restored; no irreversible damage appeared to be caused by a short borate action on the tissue. On prolonged action the cell lost the power of recovery.

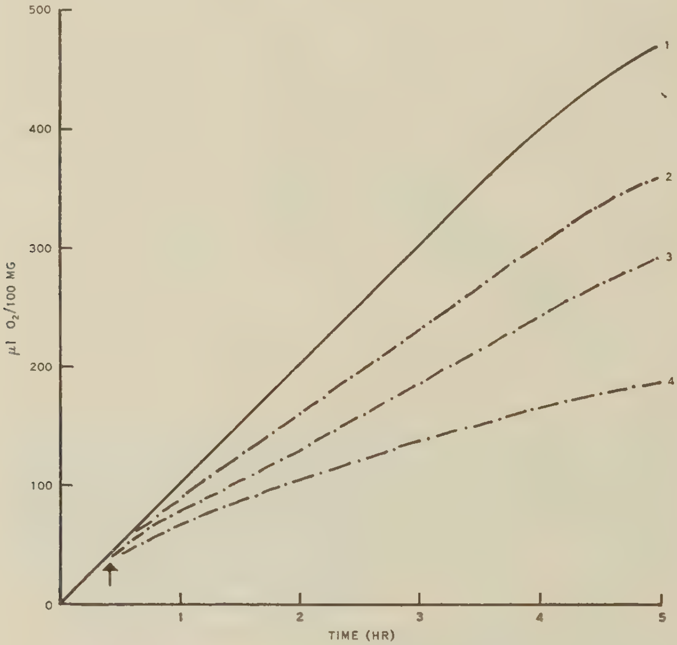
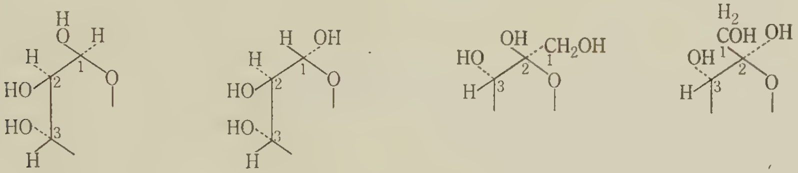


Fig. 13.—Effect of increasing concentrations of borate on the respiration in the presence of 0.005M fructose. Borate and fructose added simultaneously after 25 min. Compare Figure 10.

Curve 1: Fructose alone; no borate.
Curve 2: 0.065M borate.
Curve 3: 0.13M borate.
Curve 4: 0.26M borate.

The depressant effect of borate on the oxygen uptake of the cell may be caused through ionic reactions, or through complex formation between borate and suitable hydroxy compounds. Ionic reactions of borate may take place through formation of insoluble salts, e.g. with calcium, or by displacement, e.g. of phosphoric acid; Wiley (1904) reported increased secretion of inorganic phosphate after borate ingestion, and Pfeiffer, Hallman, and Gersh (1945) observed that, possibly through formation of glyceroborates, borate displaces phosphate in a lipid fraction of the brain. Complex formation with the substrate is suggested in the inhibition of plant polyphenolase (MacVicar and Burris 1948); complexes are formed with pyridoxine (Scudi, Bastedo, and Webb 1940) and riboflavine (Frost 1942; cf. also the review by Zittle 1951). Furthermore, with muscle adenylic acid (Klimek and Parnas 1932), dehydroascorbic acid (Militzer 1945), adrenaline (Trautner and Messer 1952), and many other hydroxy compounds, particularly sugars and intermediates of sugar metabolism —unless one or more of the HO — groups concerned is protected by phosphorylation. (According to Boeseken (1949) the complexes with sugars are mainly those involving the potential aldo and keto groups. As seen in Figure 14, only β-glucose does not possess two free adjacent HO — groups in *cis*-position and cannot therefore form borate complexes involving carbons 1, 2, or 3.) The toxic effects of borate are thus likely to be spread over a multitude of metabolic processes; the depression of the O_2 uptake in substrate-free medium is only the most striking and possibly the earliest effect of the drug.



α-glucose β-glucose α-fructose β-fructose

Fig. 14.—Steric positions of the hydroxyl groups at the 1, 2, and 3 carbon atoms of α and β glucose and fructose.

(ii) Glucose, added to the medium to a concentration above 0.01M, completely inhibited the respiratory depression caused by up to 100 times excess of borate; fructose in the same concentrations showed the same effect. Mannitol, however, although it forms complexes with borate, showed no effect even in the highest concentrations compatible with isotonic conditions. Obviously glucose and fructose do not restore the oxygen uptake by detoxicating the borate through complex formation. It is tentatively suggested that borate causes the fall in oxygen uptake initially by depriving the cell, through complex formation, of its supply of available hexose, thus accelerating the exhaustion of the cell and its final death. This interpretation accounts for the facts that (1) borate acts more slowly on the fresh cell which is still supplied with a certain store of carbohydrate, and more quickly on the aged cell whose stores are exhausted; (2) glucose counteracts the effect of a manifold excess of borate—since it is only necessary for it to raise the concentration of non-borate-combined sugar to the minimum required for continuance of normal respiration, and (iii) almost complete recovery occurs if the tissue is in time transferred into borate-free, sugar-containing medium since the unstable complexes hydrolyse on dilution. In liver, where there is an abundant supply of glycogen, borate does not cause any depression of the respiration (Feinstein and Stare 1940); the glycogen present acts like the additional glucose in our experiments and its utilization does not appear to be interfered with.

Part of the symptoms of borate poisoning in man or animals, such as convulsions and coma, may thus be due to an acute lowering of the concentration of available hexose structures in tissues which, like brain and nerve, are not supplied with a big store of carbohydrate. Pfeiffer, Hallman, and Gersh (1945) observed, however, that borate poisoning in dogs is neither prevented nor cured by the administration of glucose. It must therefore be doubted whether the maintenance of the normal respiratory rate observed in the presence of hexose indicates a normal physiological respiration. In fact, this is not the case; investigations to be presented in a following publication showed, for example, that the toxic effects of borate on the nitrogen metabolism of the cell are not counteracted by hexose. The sugars only maintain the rate of oxygen uptake, but they do not prevent borate poisoning altogether.

(iii) Low concentrations of glucose or fructose (0.001M) have in the absence of borate an identical effect on the oxygen consumption of the brain cell; in its presence, however, significant differences were observed.

(1) The maintenance concentration of fructose, i.e. the concentration which just maintained a constant oxygen consumption during the experimental time, was in the presence of borate several times higher than that of glucose (about 0.01M compared with 0.002M for glucose). Borate forms complexes involving the potential aldo resp. keto group with all fructose structures and with α-glucose, but not with β-glucose. The site of this borate effect might therefore be placed at hexokinase level, where, at low hexose concentrations, borate may demonstrate the fact that brain hexokinase, like yeast hexokinase, reacts with all glucose structures, but only with β-fructo-furanose, which constitutes only 12-20 per cent. of the fructose supplied (Gottschalk 1943; Slein, Cori, and Cori 1950). The failure of fructose satisfactorily to substitute for glucose is, however, not confined to borate action or to extreme conditions, like low ATP concentrations (Meyerhof and Geliazkowa 1947). Even in high concentrations it may not be well utilized if the cell is in any way damaged. The best known instance of this failure is the fact that fructose is inferior to glucose in relieving insulin coma. It may therefore not be justified to ascribe the lower fructose utilization in the presence of borate to complex formation alone. Other borate effects may contribute to cause the phenomenon observed.

(2) At concentrations below those required for maintenance fructose increased with increasing concentration the oxygen uptake of the borate-treated cells until, at about 0.01M, it coincided with that of normal respiration. This behaviour is to be expected, if a metabolizing system is supplied with increasing substrate concentrations. Glucose, however, caused an initial period of normal oxygen consumption before the onset of respiratory depression. It will appear that glucose, even in very low concentrations, is capable of at least temporarily supporting normal cell respiration, thus causing a delay in the onset of respiratory depression, which increased with increasing concentrations of glucose until, at about 0.002M, the oxygen uptake remained constant during the observation

time. Fructose, though causing a fairly steady oxygen uptake, never effected this temporary maintenance of the normal respiratory rate. The causes for this difference may, tentatively, be looked for at oxo-isomerase level, where fructose, as the 6-phosphate, joins the path of glucose metabolism. This enzyme requires for its function free 1, 2, 3 carbon hydroxyl groups of the reacting hexose-6-phosphates. If borate forms complexes with the 6-phosphates to the same extent as with the free sugars, the Lohmann equilibrium will be affected and at low fructose concentrations in the medium there will inside the cell result a dearth of glucose structures for those mechanisms which require glucose as substrate, like glucose-6-phosphate dehydrogenase. The question whether one of these mechanisms is concerned with the maintenance of normal brain respiration is of great interest and may have a bearing on the fact that brain is so sensitive to lowered glucose concentrations. It is, however, not considered that the facts so far known allow any more detailed interpretation of the observations presented, nor any speculations as to the nature and site of hypothetical mechanisms possibly concerned with the maintenance of normal respiration and the way in which borate may affect them.

The investigation is being extended to nitrogen and phosphate metabolism in the presence of borate.

V. Acknowledgment

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THE PERSISTENCE OF ENDOMETRIAL CYSTS INDUCED BY OESTROGEN IN GUINEA PIGS

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Summary

Cystic glandular hyperplasia of the endometrium was induced in castrate and entire guinea pigs by injecting oestradiol (15 μg per week), or by feeding them oestrogenically potent subterranean clover, for 7-8 weeks. The presence of the lesion was determined by removal of one uterine horn at biopsy from some of the animals at the end of the 7-8 weeks, after which all animals were left without treatment for a further period of 20 weeks.

In the entire animals not subjected to biopsy, cystic endometrial glands were present at the end of the second period in six out of eight animals injected with oestradiol, and in three out of five animals fed on clover, whereas all the eight undosed guinea pigs had normal uteri. The difference between dosed and undosed groups was significant ($P < 0.01$). In guinea pigs subjected to biopsy, cystic glands were found at autopsy in six out of eight undosed animals, whereas only three out of eight oestradiol-treated animals and none of the five clover-fed animals had the lesions. Again, the difference between dosed and undosed groups was significant ($P < 0.05$). The uterine horns removed from the eight undosed animals at biopsy were histologically normal.

The lesion was more severe in castrate than in entire animals. This was particularly noticeable in the clover-fed groups. Significant regression of the lesions between biopsy and autopsy occurred in the non-ovariectomized, oestrogen-treated groups, but not in the ovariectomized, oestrogen-treated groups.

Observations were also made on oestrous periods and nipple lengths during the experiment, and on uterine size and the gonadotrophic content of the pituitary glands at the end of the experiment. Oestrus, as judged by the patency of the vagina, was continuous during the oestrogen treatment. Soon after the cessation of treatment, normal oestrous cycles were resumed in 11 of the 13 animals not subjected to biopsy or ovariectomy.

It is concluded that endometrial glands which become cystic under the influence of oestrogen can remain in this condition for long periods after the cessation of oestrogenic stimulation, and in the absence of a persisting endocrine imbalance.

I. INTRODUCTION

Cystic glandular hyperplasia of the endometrium is a common sequel to oestrogen treatment in the mammalian female. The lesion has been reported following a course of oestrogen injections in guinea pigs and rats (Wolfe,

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Campbell, and Burch 1932), women (Kaufmann 1934), mice (Parkes 1935), monkeys (Zuckerman and Morse 1935), rabbits (Lacassagne 1935), ground squirrels (Wells and Overholser 1940), and golden hamsters (Bacon and Kirkman 1949). However, until recently the question of the persistence of the lesion after the cessation of oestrogen treatment had received little attention. Cystic endometrial hyperplasia, induced in monkeys by oestrogen treatment, was found to regress within 2-4 months after the termination of the treatment (Dahl-Iversen, Hamburger, and Jorgensen 1942). On the other hand, the condition is known to be present in post-menopausal women for long periods in the apparent absence of oestrogenic stimulation (Novak and Richardson 1941).

In 1946, Bennetts, Underwood, and Shier reported a breeding problem in sheep depastured on subterranean clover. The manifestations were cystic glandular hyperplasia of the endometrium, infertility, and, in those that did conceive, dystocia. Strong evidence that the syndrome resulted from the presence of an oestrogenic substance in the clover was presented by these workers and by Curnow, Robinson, and Underwood (1948), and Robinson (1949). Bradbury and White (1951) and Curnow (personal communication 1952) identified this substance as the isoflavone genistein, which they isolated from extracts of clover. It was found that the cystic endometrial hyperplasia and the infertility remained for several years after the affected animals were transferred to non-clover pastures (Schinckel 1948; Underwood and Shier 1951). However, there was no concomitant disturbance of the oestrous cycles of affected sheep (Bennetts, Underwood, and Shier 1946; Underwood and Shier 1952).

These reports suggested that, in one species at least, endometrial cysts, induced by exogenous oestrogens, can persist for long periods in the absence of excessive oestrogenic stimulation or endocrine imbalance. The present study was undertaken in an endeavour to establish whether or not this applied to laboratory animals as well. The effect of oestradiol treatment was compared with clover feeding to discover whether the action of the clover oestrogen differed from that of oestradiol.

II. Methods

Sixty-one virgin female guinea pigs, about 3 months old, were randomized into eight groups, five containing eight animals each and three containing seven.

The experiment entailed a period of oestrogenic stimulation lasting 7-8 weeks, followed by a second period of about 20 weeks without any treatment. At the end of the first period biopsies were performed on groups 1, 3, 4, 6, and 7, the greater part of the free portion of the left uterine horn being removed. At the conclusion of the second period, all the animals were killed by chloroform and tissues were removed for histological examination. The treatment of the individual groups is shown in Table 1.

The subterranean clover used was collected (in 1945 and 1946) when green, dried immediately, and kept in airtight tins. It was finely ground and fed *ad lib.* to guinea pigs in groups 1, 2, and 3. In these groups ascorbic acid was added to the drinking water during the period for which the animals were fed on clover. Three weeks after the start of the experiment a supplement was added to the clover as all the clover-fed animals were losing weight and three deaths had occurred. Normal weight gains were maintained on the supplemented ration which consisted of clover, 80 per cent.; pollard, 10 per cent.; meatmeal, 5 per cent., and bakers' yeast, 5 per cent. The remaining groups were fed on a normal diet which included fresh lucerne. Oestradiol benzoate was administered intramuscularly in arachis oil to groups 4, 5, and 6 during the first 7 weeks of the experiment. Doses of 5 μg oestradiol benzoate were given three times weekly. The animals in groups 3 and 6 were subjected to ovariectomy 2 days prior to the start of the experiment. During the second period no oestrogen treatment was given and all the animals were fed on the normal diet. Body weights were recorded weekly, and nipple lengths measured with vernier callipers every 2 weeks. Oestrus, as exhibited by vaginal patency, was recorded daily.

Table 1

TREATMENT OF GUINEA PIGS IN GROUPS 1-8

Group Number	Number of Animals	Ovaries Removed	Oestrogen Treatment	Biopsy at 8 Weeks
1	8	–	Subterranean clover	+
2	7	–	Subterranean clover	–
3	7	+	Subterranean clover	+
4	8	–	Oestradiol benzoate	+
5	8	–	Oestradiol benzoate	–
6	7	+	Oestradiol benzoate	+
7	8	–	None	+
8	8	–	None	–

All the tissues examined, except the pituitary glands, were fixed in 10 per cent. formalin followed by Vandergrif's fixative. Paraffin embedded sections were stained by haematoxylin and eosin. The pituitary glands were fixed for 2 hr in 10 per cent. neutral formol saline followed by overnight fixation in Zenker-formol and paraffin embedding. Sections from 45 pituitaries were stained by the method described by Pearse (1950), which involves the periodic acid-Schiff technique. With a 1.8 mm objective and an 8X ocular about 10 fields were counted from each pituitary. The numbers of cells staining positively by the Schiff stain were expressed as a percentage of the total numbers of cells, and the results analysed statistically.

Uterine size measurements.—A comparison of uterine size could not be made on the basis of weight as biopsies had been performed in some groups. However, differences in the size of the organ between groups were conspicuous, so the area of a transverse section through the middle of the body of the uterus was used to provide an objective basis for comparison. Stained sections of the uteri were projected onto ruled paper at a magnification of about 5.5X, and their outlines were recorded.

III. Results

(*a*) *Nipple Length*

The initial mean nipple length of all groups was 3.0 mm. The nipple length of the control groups remained unaltered for approximately 12 weeks. During the next few weeks an increase in length of about 30 per cent. occurred, after which no further growth took place.

Table 2

EFFECT OF THE SURGICAL AND OESTROGENIC TREATMENTS ON THE OESTROUS CYCLES OF GUINEA PIGS

Group Number	Treatment	Period 1 (about 8 weeks)	Period 2 (about 20 weeks)
1	Clover and biopsy	Continuous oestrus after third week	Vaginae of all 5 animals closed in 3-18 days, after which oestrus was irregular. After 2-4 cycles 3 animals remained in anoestrum to end of experiment (105-138 days)
2	Clover	Continuous oestrus after third week	Vagina remained open for 5 weeks in one animal, in 4 others it closed within 4 days. Normal oestrous cycles thereafter
3	Ovariectomy, clover, and biopsy	Continuous oestrus within 1 week	Vaginae closed within 1 week. Anoestrum to end of experiment
4	Oestradiol and biopsy	Continuous oestrus within 5 days	Normal cycles resumed in the 8 animals 5-12 days after last injection. Two showed some irregularity in not exhibiting oestrus for up to 66 days after several normal cycles
5	Oestradiol	Continuous oestrus within 1 week	Six animals resumed normal cycles in 5-17 days after last injection. Two were abnormal: vaginal closing occurred in one animal after 60 days followed by normal cycles; in another the vagina remained open for 115 days, then for the remaining 4 weeks opening and closing occurred irregularly every few days.
6	Ovariectomy, oestradiol, and biopsy	Continuous oestrus within 3 days	The vaginae of all 6 animals closed 3 days after last injection. Irregular opening and closing for next 32 days, followed by anoestrum to end of period 2
7	Biopsy only	Normal cycles	Four guinea pigs had normal cycles. Two had two long periods with vagina closed up to 50 days, at the beginning of period 2, and then returned to normal. Another showed oestrus twice only and another once only
8	No treatment	Normal cycles	All 8 animals had normal cycles

The three clover-fed groups showed increases in mean nipple length ranging from 60 to 70 per cent., commencing shortly after the beginning of feeding and reaching a maximum within 6 weeks, except among the ovariectomized animals in group 3, in which the maximum was reached in about 4 weeks. Some regression occurred during the remainder of the treatment period in this group; the mean nipple length at the end of the feeding period was only 25 per cent. above the initial value. After the cessation of clover feeding, however, the nipple length in groups 1 and 2 dropped within a fortnight to the same level as group 3. After this, the mean nipple length in group 3 declined gradually to about 10 per cent. greater than the initial length. The value in group 2 remained unaltered, whereas in group 1 it gradually increased again to about 60 per cent. above the initial length.

In the three groups treated with oestradiol, the increase in mean nipple length was more rapid and greater than in the clover groups. The maximum increase attained was 75-90 per cent. After the cessation of treatment, there was a rapid regression, but not to the initial levels. The mean nipple lengths in groups 4, 5, and 6 at the end of the experiment were respectively 30, 50, and 20 per cent. greater than the initial length.

(*b*) *Oestrus*

In the control animals (group 8) oestrous cycles were regular and oestrus lasted 3-5 days. The period of vaginal closing between successive oestrous periods was generally between 13 and 18 days. Occasional oestrous periods were not detected, so that the vagina apparently remained closed for about 30-33 days, but this could be caused either by failure of the vagina to open or by a very short oestrus. The results are summarized in Table 2.

TABLE 3

MEAN LOG CROSS-SECTIONAL AREA OF GUINEA PIG UTERI FOLLOWING VARIOUS TREATMENTS

Treatments	Biopsy		Non-biopsy		Ovariectomy	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
Clover	2·17	0·067	1·87	0·067	1·49	0·067
Oestradiol	1·95	0·053	2·13	0·053	1·60	0·061
Undosed	1·98	0·053	1·81	0·056		

(*c*) *Uterine Size*

The mean log cross-sectional area of the uterus in each group and its standard error are set out in Table 3. The variances of the uterine size within each group increased with the means of the groups, so analyses were carried out on logarithms (see Table 4). Because there was no undosed, ovariectomized group, two analyses of variance were carried out—one including two surgical treatments (biopsy and non-biopsy) with three oestrogenic treatments (clover, oestradiol, and undosed), and the other including three surgical treatments and two oestrogenic treatments (clover and oestradiol).

The first analysis indicated a significant interaction between surgical and oestrogenic treatments, arising from the fact that the value for the biopsied animals was lower than that for the non-biopsied animals in the oestradiol-treated groups, whereas in the undosed and clover-fed groups the entire animals had larger uteri than the biopsied. The high value for the clover-fed, biopsied animals was largely due to distended uterine lumens, but there seemed to be actual hypertrophy of the uterine tissue itself in the oestradiol-treated, non-biopsied group.

Table 4

ANALYSES OF VARIANCE ON UTERINE SIZE DIFFERENCES BETWEEN GROUPS

Source of Variation	Analysis 1 (omitting ovariectomized groups)			Analysis 2 (omitting undosed groups)		
	Degrees of Freedom	Mean Square	F	Degrees of Freedom	Mean Square	F
Surgical treatments	1	0·0148	—	2	0·1585	5·30
Oestrogenic treatments	2	0·0124	—	1	0·0036	—
Interaction	2	0·0306	9·01**	2	0·0299	4·43
Within groups	35	0·0034†		31	0·0067†	

** = $P<0{\cdot}01$.

† = Within groups variance divided by the harmonic mean of group numbers.

(*d*) *Histological Examination of the Uteri*

Sections of the horns and the body of the uterus were examined for the presence of endometrial cysts, and scores were given to each according to the severity of the lesion (Table 5):

−	Indicates normal endometrial histology (Plate 1, Fig. 1).
+	Indicates the presence of early glandular hypertrophy with an occasional small cyst showing flattening of the lining epithelium (Plate 1, Fig. 2).
++	Indicates the presence of a number of medium-sized cysts (Plate 1, Fig. 3).
+++	Indicates a severe lesion characterized by numerous large cystic glands, as depicted in Plate 1, Figure 4. The flattened epithelial lining of such glands is illustrated in Plate 2, Figure 5.

As the number of animals in each group was small, the scoring system was simplified for statistical anlysis. The animals were grouped into two classes: those with normal uteri (scored −), and those with abnormal uteri (scored +, ++, or +++). For the comparison of the scores of the uterine horns removed at biopsy (Table 5, column *A*), χ^2 tests were carried out. The χ^2 value for groups 1, 4, and 7 was found to be 19.402, which is highly significant ($P < 0.001$), and results from the fact that all the undosed animals (group 7) had normal uteri, whereas none of the oestradiol-treated animals and only one of the seven clover-fed animals had normal uteri. There was no difference between the two ovariectomized groups at biopsy, though there was a tendency for the lesions to be more severe in the clover-fed than in the oestradiol-injected animals.

In order to determine whether any regression of the lesions took place between biopsy and slaughter 20 weeks later, the horn and body of the uterus were scored separately in each animal (see columns *B* and *C*, Table 5). However, for comparisons between treatments, the scores of the two parts were combined to give a score for each uterus as a whole. This was done by taking the higher of the two scores as representing the whole uterus. In all except group 7, these combined scores correspond to those given for the body only in column *C* of Table 5. In group 7, the numbers of animals under each classification were −2, +3, ++3, +++0. Separate χ^2 tests on the combined scores were done for the biopsied animals (groups 1, 4, and 7) and the non-biopsied animals (groups 2, 5, and 8). There were significant differences in the numbers of animals with the lesions between the three groups subjected to biopsy ($\chi^2 = 7.219$, $P < 0.05$) and also between the three groups not subjected to biopsy ($\chi^2 = 9.551$, $P < 0.01$). In the biopsied groups, only two out of eight undosed animals (group 7) had normal uteri, whereas all the clover-fed animals (group 1) and five out of eight oestradiol-injected animals had normal uteri. In the non-biopsied groups, on the other hand, all the undosed animals had normal uteri, whereas only two out of eight oestradiol-treated and three out of five clover-fed animals had no lesions. There was little difference between the scores for the two ovariectomized groups. The differences in the combined

TABLE 5

EFFECT OF SURGICAL AND OESTROGENIC TREATMENTS ON THE DEVELOPMENT AND PERSISTENCE OF CYSTIC ENDOMETRIAL HYPERPLASIA IN GUINEA PIGS. NUMBERS INDICATE THE NUMBER OF ANIMALS WITHIN EACH CLASSIFICATION

Group Number	Treatment	*A* Uterine Horn at Biopsy				*B* Uterine Horn at Autopsy				*C* Uterine Body at Autopsy			
		−	+	++	+++	−	+	++	+++	−	+	++	+++
1	Clover, biopsy	1	4 (2)*	2	0	5	0	0	0	5	0	0	0
4	Oestradiol, biopsy ..	0	2	6	0	7	0	1	0	5	1	2	0
7	Undosed, biopsy ..	8	0	0	0	3	2	3	0	4	1	3	0
2	Clover, non-biopsy ..					4	1	0	0	3	2	0	0
5	Oestradiol, non-biopsy ..					3	2	3	0	2	0	6	0
8	Undosed, non-biopsy ..					8	0	0	0	8	0	0	0
3	Clover, ovariectomy, biopsy	0	0	4 (1)*	3 (1)*	0	1	3	1	0	0	1	4
6	Oestradiol, ovariectomy, biopsy	0	2 (1)*	5	0	3	2	1	0	0	0	1	5

* These figures in brackets indicate animals included in that classification which later died, and are therefore not included in subsequent totals.

+ To +++ indicates increasing severity of cystic hyperplasia.

− Indicates normal histology. See Plate 1, Figures 1-4.

scores for the whole uterus between groups 2 and 3, and between groups 5 and 6 (see column *C*, Table 5) suggest that ovariectomy resulted in an increase in the severity of the cystic endometrial lesion. This was particularly evident in the clover-fed animals.

The comparisons between the horn scores at biopsy and at slaughter (Table 5, columns *A* and *B* respectively) were carried out by χ^2 tests, again grouping the animals into two classes—those with, and those without, cystic endometrial glands. For groups 1, 4, and 7 there were significant differences between the numbers without lesions at biopsy and at slaughter. The χ^2 values found were: clover-fed group, 5.486 ($P < 0.05$); oestradiol-treated group, 9.143 ($P < 0.01$), undosed group, 4.655 ($P < 0.05$), and all three groups together, 19.284 ($P < 0.001$). In the oestrogen-treated animals (groups 1 and 4) there was an almost complete regression of the lesions during the 20 weeks between the cessation of oestrogen treatment and the end of the experiment, but in the undosed animals the condition had developed in five out of eight animals, all of which had normal uteri at biopsy, as judged by the condition of the horn removed. The blood supply to the ovary was not damaged by the biopsy operation. There was no significant regression of the lesion between biopsy and the end of the experiment in the castrates.

TABLE 6

MEAN NUMBERS OF P.A.S. REACTIVE CELLS AS A PERCENTAGE OF THE TOTAL NUMBER OF CELLS IN THE PITUITARY GLANDS OF GUINEA PIGS IN GROUPS 1-8

Group Number	1	2	3	4	5	6	7	8
Percentage of cells stained by P.A.S.	43·8	36·2	45·1	40·3	38·2	50·3	42·3	41·6

Standard deviation = 3·7.

(*e*) *Examination of the Pituitary Glands*

A total of 1000-2000 cells was counted in each pituitary, and the number of cells stained by the periodic acid-Schiff (P.A.S.) technique was expressed as a percentage of the total number of cells. The only pituitary hormones which are known to be glycoproteins are the gonadotrophins and thyrotrophin and, as the latter is thought to have a much smaller proportion of carbohydrate in the molecule, the intensity of staining by the P.A.S. technique may be taken as some indication of the amount of gonadotrophin present. Pearse and Rinaldini (1950) have shown this to be so in starved rats.

The mean values for the number of cells stained by the P.A.S. technique expressed as a percentage of the total number of cells are given in Table 6. Because there was no ovariectomized, undosed group, two analyses of variance were carried out — one including two surgical treatments (biopsy and non-biopsy) with three oestrogen treatments (clover, oestradiol, and undosed), and the other including three surgical treatments and two oestrogenic treatments (clover and oestradiol). These analyses are given in Table 7. The analyses revealed that the effect of the surgical treatments, and the interaction of surgical and oestrogenic treatment, was significant. Of the surgical treatments, ovariectomy (groups 3 and 6) induced a higher percentage of P.A.S. positive cells than biopsy (groups 1, 4, 7; $P < 0.01$), which in turn induced more P.A.S. positive cells than non-biopsy (groups 2, 5, 8; $P < 0.01$). The pituitary glands of animals subjected to castration and oestradiol treatment (group 6) had a significantly higher percentage of P.A.S. positive cells than the castrated, clover-fed animals (group 3; $P = 0.05$).

TABLE 7

ANALYSES OF VARIANCE ON THE NUMBERS OF P.A.S. REACTIVE CELLS IN THE PITUITARY GLANDS OF TREATED GUINEA PIGS

Source of Variation	Analysis 1 (omitting ovariectomized groups)			Analysis 2 (omitting undosed groups)		
	Degrees of Freedom	Mean Square	F	Degrees of Freedom	Mean Square	F
Oestrogenic treatments	2	3·9005	1·605	1	2·2448	—
Surgical treatments	1	18·5153	7·619*	2	55·2604	19·912***
Interaction	2	6·7763	2·788	2	9·7331	3·507
Within groups	28	2·4302		26	2·7752	

*** = P<0·001; * = P<0·05.

IV. DISCUSSION

The results of the present investigations indicate that, in the guinea pig, cystic glandular hyperplasia of the endometrium, induced by oestrogen administration, can persist for long periods after the cessation of treatment. The animals thus affected usually exhibit normal oestrous cycles. Similar effects following oestrogen administration to mice have been reported elsewhere (Braden 1952). In guinea pigs, the presence of ovaries is not essential to the production and persistence of the cystic glands; indeed, the condition is more easily induced in ovariectomized than in entire animals. Novak and Richardson (1941) report the persistence of cystic endometrial glands over long periods in post-menopausal women with little evidence of oestrogenic stimulation in the rest of the endometrium. Their observations, as well as those on sheep (Bennetts, Underwood, and Shier 1946) and the present results, indicate that the glands are not maintained in their cystic condition by an endocrine imbalance, which persists after the cessation of oestrogen administration or secretion, but that the cysts are autonomous. In accord with this hypothesis are the observations that many animals with cystic endometrial hyperplasia exhibit normal oestrous cycles, and that oestrogen treatment has no significant effect on the subsequent histological picture of the pituitary. The mechanism by which the glands are maintained in the cystic condition may be mainly mechanical.

The position in pre-menopausal primates appears somewhat different, however. In women before the menopause, cystic endometrial hyperplasia is almost invariably associated with ovarian abnormalities (Schroeder 1915; Fluhman 1931), but when normal ovarian function is restored or the ovaries are removed, the lesion disappears (Taylor 1944; Wolfe 1950). The difference in this respect between primates and other mammals is probably related to the difference between their endometrial cycles; in primates the major part of the endometrium containing the cystic glands would probably be shed during menstruation.

The cystic endometrial hyperplasia induced in guinea pigs by feeding subterranean clover is presumably a manifestation of the action of genistein, the only substance possessing oestrogenic activity known to be present in the clover in significant amounts (Bradbury and White 1951; Curnow, personal communication 1952). It appears unlikely from the present results that the mode of action of this oestrogen differs from that of oestradiol. The differences between the effects of clover feeding and oestradiol injection can probably be imputed entirely to a lower oestrogenic dose level in the former. In support of this are the observations that the oestradiol treatment caused a greater increase in nipple length than the clover feeding, and also induced continuous oestrus sooner.

In relation to the breeding problem in sheep depastured on subterranean clover (Bennetts, Underwood, and Shier 1946), the present observation that ovariectomy increases the effectiveness of oestrogenic treatment in inducing the cystic glandular condition, is of significance. Apparently the oestrogenic stimulus need be little above the threshold required for glandular response, as the clover feeding produced few permanent cysts in non-castrate guinea pigs. In comparison with the more common oestrogens, the potency of genistein is low (Bradbury and White 1951); even a diet consisting almost entirely of fresh, potent subterranean clover would represent only a relatively low oestrogen dosage. What seems to be of more importance is the fact that the sheep are normally in anoestrum during the months they are grazing the potent clover. During this period there would be little circulating progesterone to counteract the effects of the ingested oestrogen. Korenchevsky and Hall (1940) find that the concurrent administration of progesterone prevents the appearance of cystic glandular hyperplasia in the uterus of spayed rats treated with oestrogen and androgen. Likewise, Taylor (1944) concludes that a great excess of oestrogenic stimulation is not the essential factor in inducing the lesion, but rather a continuity of oestrogenic stimulation without the periods afforded by the normal oestrous cycle when the effect of oestrogen is counteracted by that of progesterone. However, very little cystic hyperplasia was found by Bennetts and Underwood (1951) in ovariectomized ewes grazed on subterranean clover during the 1947 and 1948 seasons. The absence of severe lesions in these ewes may have been the result of a lower intake of oestrogen than affected sheep received in the years when the incidence of the condition was highest (1941-1945). Recent evidence indicates that the oestrogenic potency of the clover was considerably greater during those years (Alexander and Rossiter 1951).

In contrast to the present results, East (1952) apparently found little or no cystic glandular hyperplasia in the uteri of guinea pigs maintained on a clover diet, or injected with oestradiol, for 6-9 months. Hyperplasia, metaplasia, and keratinization of the epithelium of the genital tract were present, however, and considerable, though not complete, regression of these conditions occurred during a further period without treatment. It would appear that the effective oestrogen dose rate in most of her groups was less than in the present study, as she did not observe continuous oestrus except in animals on the highest dose rate. In these animals, ovarian atresia was still present at the end of the experiment.

The removal of one horn of the uterus in some of the groups introduced a complication. This procedure, in the absence of oestrogen administration, induced cystic endometrial hyperplasia in most of the animals. However, when the operation was performed on oestrogen-treated animals the number which subsequently exhibited the lesion was less than in the groups in which the animals were subjected to either treatment alone. An interaction between surgical and oestrogenic treatments was also found in the analyses of estimates of uterine size, and of the gonadotrophin content of the pituitary glands, but the mechanism of this interaction is obscure.

As far as is known, cystic glandular hyperplasia of the endometrium as a sequel to subtotal hysterectomy has not been reported before. However, the condition may not develop as a result of the removal of part of the uterus in other species, as it has been observed that, in three rabbits treated in this way, the remaining uterine horn appeared quite normal 4 months later (Braden 1952). Cystic glandular hyperplasia of the endometrium has been noticed in several species following surgical procedures involving removal of a part of the ovary or pituitary. Thus subtotal ovariectomy in rats, guinea pigs, and cats (Burch, Wolfe, and Cunningham 1932; Lipschutz 1937) or subtotal hypophysectomy in guinea pigs will produce the condition (Burch *et al.* 1937). Clinical observations indicate that women are similarly affected by ovarian resection (Burch, Wolfe, and Cunningham 1932) or ovarian autotransplantation (Knudtzon 1937). However, Dahl-Iversen, Hamburger, and Jorgensen (1942) and Van Wegenen and Morse (1942) could not produce the lesion in monkeys by the two last-mentioned techniques. A possible explanation of the effect of these surgical procedures in inducing cystic glandular hyperplasia is that they permanently upset a delicate hormonal balance between the pituitary, ovaries, and uterus. In support of this hypothesis is the present finding that some of the animals from which one uterine horn had been removed displayed irregular oestrous cycles. Also, the present results suggest that subtotal hysterectomy increases the gonadotrophic activity of guinea pig pituitary glands.

V. ACKNOWLEDGMENTS

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Explanation of Plates 1 and 2

Plate 1

Fig. 1.—Normal guinea pig uterus, classification — (see text) (guinea pig No. 1, group 7). Haematoxylin and eosin. × 38.

Fig. 2.—Guinea pig uterus, classification + (guinea pig No. 4, group 5). Haematoxylin and eosin. × 38.

Fig. 3.—Guinea pig uterus, classification ++ (guinea pig No. 8, group 4). Haematoxylin and eosin. × 38.

Fig. 4.—Guinea pig uterus, classification +++ (guinea pig No. 7, group 3). Haematoxylin and eosin. × 38.

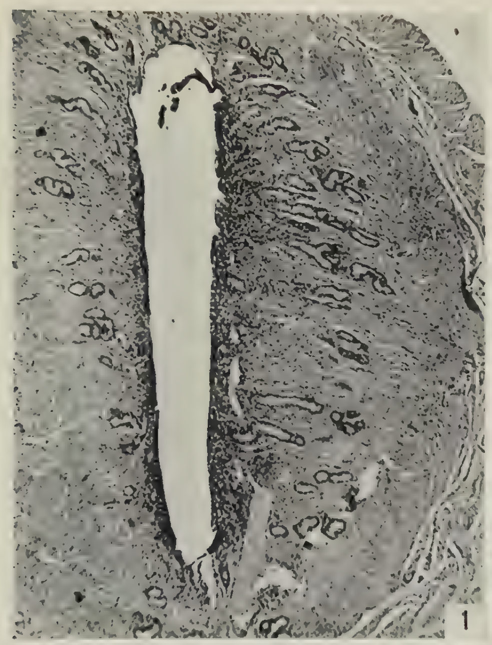
PLATE 2

Fig. 5.—Portion of the uterus illustrated in Figure 4, showing the flattened glandular epithelium lining one of the large cysts. Haematoxylin and eosin. × 480.

Fig. 6.—Portion of the uterus illustrated in Figure 4, showing atrophic uterine glands. Note also portion of a normal gland and the cubical epithelium lining the lumen of uterus. Haematoxylin and eosin. × 480.

Fig. 7.—Normal guinea pig uterine gland. Haematoxylin and eosin. × 480.

PERSISTENCE OF ENDOMETRIAL CYSTS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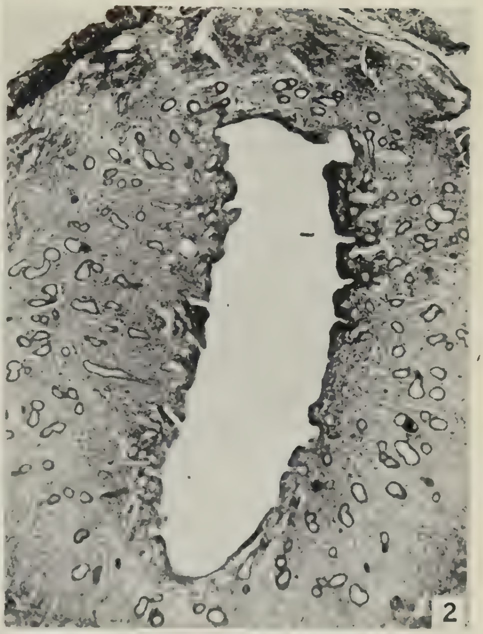
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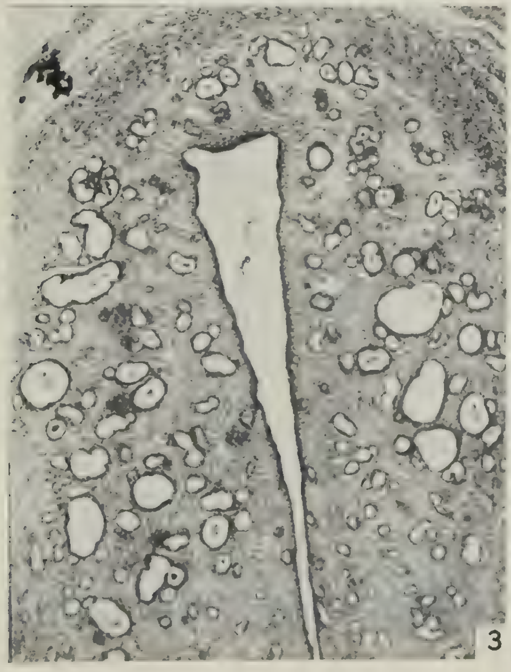
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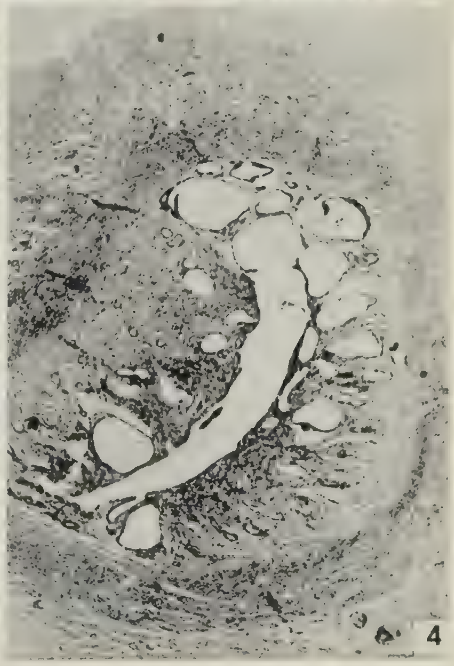
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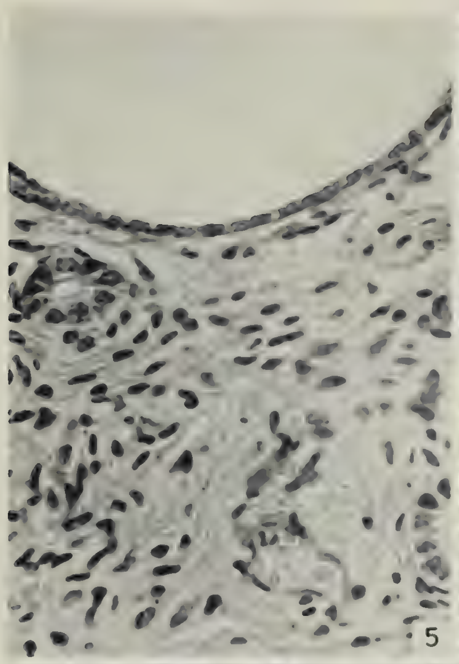
PERSISTENCE OF ENDOMETRIAL CYSTS



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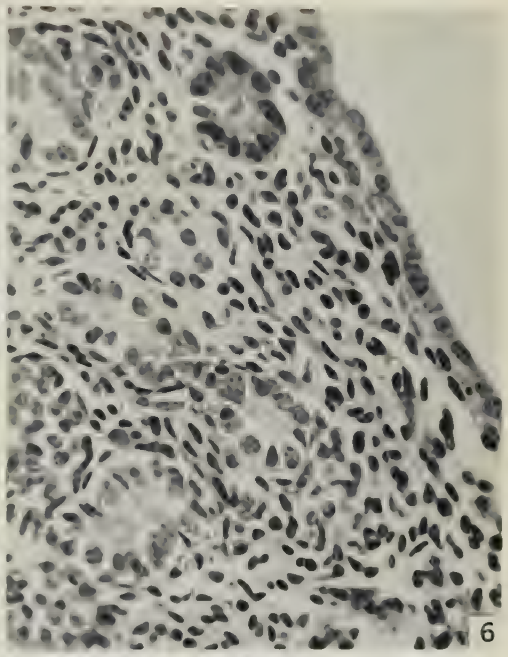

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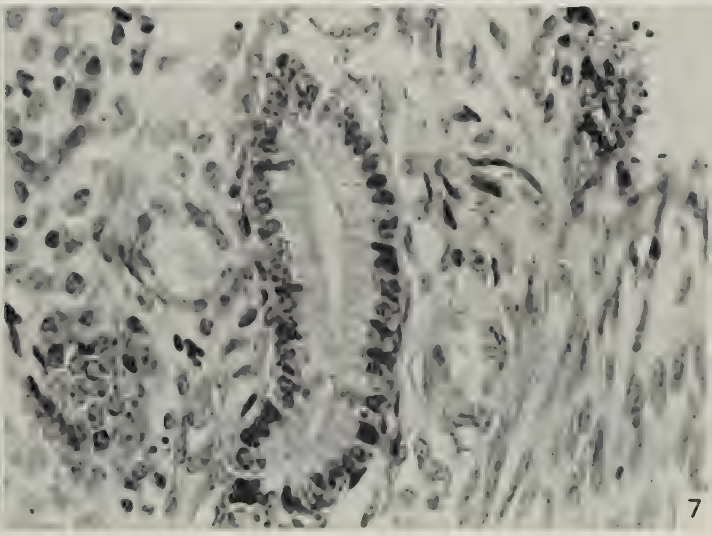

7

SKIN TRANSPLANTATION IN THE FOETAL LAMB

By P. G. SCHINCKEL* and K. A. FERGUSON†

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Summary

Skin autografts and homografts have been performed in foetal lambs between the ages of 80 and 117 days. By means of histological examination following grafting, visual and histological examinations after birth, and by the use of second-set homografts, it was established that homografts are actively rejected by the foetus. This rejection gave every indication of belonging to the general class of activity acquired immune responses and in all respects confirmed at the foetal stage the observations of Medewar (1944, 1945) on young and adult animals.

Homografts in which the ewe was used as donor were also rejected by the foetus, indicating that the reaction was of foetal and not maternal origin.

The experiments established that the foetal lamb is capable of making an immune response to the presence of foreign tissue. This finding is at variance with current concepts of the immunological behaviour of foetuses.

I. INTRODUCTION

It seems probable that skin transplantation techniques will be useful in obtaining a clearer understanding of some of the basic biological phenomena concerned in wool-fibre production. For numerous purposes we are interested in the possibility of establishing skin homografts, i.e. grafts from one animal to another, between individuals of normal genetic diversity. However, it has been clearly shown that such transplantations are not possible in normally diverse populations of human beings (Gibson and Medewar 1943), rabbits (Medewar 1944, 1945), cattle (Anderson *et al.* 1951), and guinea pigs and mice (Medewar, personal communication 1952).

The non-acceptance of such skin homografts has been shown unequivocally (Medewar 1944, 1945) to be due to an immunological mechanism of the general type of actively acquired immune reactions. However, evidence has been presented from immunological studies with chick embryos (Grasset 1929; Burnet 1941), which indicates that the developing foetus is incapable of producing antibodies and might therefore be expected to accept foreign tissues.

Further, it has been shown (Ferguson, Stormont, and Irwin 1942; Stormont and Cumley 1943) that, despite the large number of red-cell antigens known to exist in cattle, there is an abnormally high proportion of identical red-cell antigens in dizygotic twins, whereas identity is rare in full sibs. This is almost certainly a result of the synchorial nature of the foetal membranes

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in a high proportion of such twins, one effect of which is the sterile "freemartin" female born twin to a bull. As a result of the synchorial arrangement, vascular anastomoses are established leading to the interchange of red cells and red-cell precursors between the twins during prenatal life (Owen 1945; Owen, Davis, and Morgan 1946). Such red-cell precursors act as a homograft, giving rise to erythrocytes antigenically "foreign" to the host for the remainder of prenatal life and at least for the youthful part of postnatal life. Such dizygotic twins are therefore genetic chimaeras and contain cellular material genetically different from that acquired at fertilization. It has also been shown (Anderson *et al.* 1951) that skin grafts may be exchanged between the members of dizygotic cattle twins with a high degree of success. This is not so for full sibs born at different parturitions. It must be concluded from these observations that the dizygotic cattle twins are no longer able to recognize the "non-self" (Burnet and Fenner 1949) components of the cellular antigens of their twin sib.

It might be expected therefore that skin homografts transplanted during foetal life would have a reasonable chance of being accepted and of continuing normal development. Additionally, if the grafts were accepted at the foetal stage, then the indications are that further grafts would be accepted subsequently from the same donor during postnatal life.

The object of these observations was to determine whether or not skin homografts would succeed at the foetal stage in sheep.

II. Experimental Plan

(*a*) *General*

Sixteen pregnant merino ewes, with known conception dates, were used. With the exception of one ewe, 20G210, which was mated to a Border Leicester ram, all ewes were mated to a single Merino ram. The genetic composition of the population was therefore considerably more uniform than that of the rabbits used by Medewar (1944).

In the interests of economy of sheep it was planned to operate on two cases at a time in order to make reciprocal homografts (type *C*, Billingham and Medewar 1951) between the foetuses. Concurrently, autografts were performed on each foetus (with one exception) to act as controls for the homografts. On two occasions, one member of the pair was found to be non-pregnant when the abdominal cavity was opened. In each of these cases, the homografts were made from the abdominal wall of the ewe to the foetus. If a homograft reaction was to be encountered, these two cases provided the opportunity to determine whether it was primarily of foetal or maternal origin. Current concepts of placental transmission in the ewe make it extremely unlikely that the dam could be responsible for any such reaction, but on general grounds it seemed wise to explore this possibility.

Table 1 shows the general plan of the observations. These were planned primarily to determine whether skin homografts would succeed at the foetal stage. At the same time, it seemed desirable to obtain information on (1) the effect of age generally with donor and recipient of approximately the same age, and (2) the effect of differences in age between donor and recipient on the success or otherwise of the transplants (Table 1). Previous observations (unpublished data) by us indicated that it was possible to make a surgical penetration of the gravid uterus in ewes between the 110th and 143rd days of gestation (normal period 149 days) and to have normal lambs delivered at term. While it was considered desirable to extend the observations into earlier stages of pregnancy in the current series, we were unaware of the surgical risks involved and decided not to attempt transplantation before the 80th day.

TABLE 1

GENERAL PLAN OF OBSERVATIONS

Pair No.	Ewe No.	Operation Date	Days Pregnant	Age of Grafts at Biopsy*		Remarks
				A	*H*	
1	7F421	29.iv.52	80	n.r.	63	Homograft from ewe. Lamb born 9.vii.52. Lamb No. 38S8
	646		—	—	—	Not pregnant
2	27H516	30.iv.52	106	—	—	Aborted 11.v.52
	12G14		110	21	21	P.M. 21.v.52 for histological examination
3	13F163	1.v.52	106	81	54	Lambed 11.vi.52: lamb no. 38S1; repeat grafted postnatal
	16F323		107	47	47	Lambed 12.vi.52; lamb no. 38S2: repeat grafted postnatal
4	12H75	2.v.52	97	80	n.r.	Lambed 22.vi.52: lamb no. 38S3; repeat grafted postnatal
	16F371		98	n.r.	53	Lambed 21.vi.52: lamb nos. 38S4 (grafted), 38S5: repeat grafted postnatal
5	20G210	3.v.52	90	207	207	Homograft from ewe: lambed 26.vi.52: lamb no. 38S7
	13G342		—			Not pregnant
6	10F34	5.v.52	98	176	52	Lambed 25.vi.52: lamb no. 38S6
	20J10		91			Ewe died 11.v.52 from herniation of section of small intestine between sutures in abdominal wall
7	32J245	6.v.52	116	9	9	P.M. 15.v.52 for histological examination
	32J242		116	15	15	P.M. 21.v.52 for histological examination
8	16G136	8.v.52	117	22	22	P.M. 30.v.52 for histological examination
	12H69		115	15	15	P.M. 23.v.52 for histological examination
9	16G101	8.v.52	85	n.r.	n.r.	Lambed 10.vii.52: lamb no. 38S9
	16F365		117	33	33	Lambed 10.vi.52: lamb died at birth

* *A* = autograft; *H* = homograft; n.r. = not removed.

In order to obtain information on the histological changes associated with the grafts, five ewes were killed at post-operative intervals of 9, 15 (2 ewes), 21, and 22 days. The first autopsy in this series was later than that used by Medewar (1944, 1945) in adult rabbits (4 days) but the decision to commence at 9 days was based on the hypothesis that if a homograft reaction was to be encountered it would probably be of a lower order than that noted by Medewar. We now know this to be incorrect and feel sure that a greater amount of information would have been gained had autopsy examinations commenced much earlier.

The remaining ewes were allowed to proceed to term. Soon after parturition each lamb was closely inspected, the operation site located, and observations recorded on the condition of the grafts. Some were removed at this time and others allowed to remain for further observations at later periods. In one lamb (38S7 from ewe 20G210) the grafts were not located until the lamb was killed at 5 months of age and the entire skin thoroughly examined.

Where possible, reciprocal homografts were repeated between available lambs during postnatal life. The details of these transplants are presented later.

(*b*) *Surgical Procedure*

Anaesthesia was induced and maintained throughout the operations with either "Kemithal" (I.C.I.) or "Nembutal" (Abbott) administered intravenously. All incisions were made with the use of a diathermy unit.

The abdominal wall was prepared with 1 per cent. aqueous "Cetavlon" (I.C.I.) after close clipping and opened with a 4-5 in. paramedian incision, commencing about 2 in. in front of the mammary gland. The uterus was readily located and brought to the site of the abdominal incision, but it was not exteriorized. By gentle manipulation and the use of sterilized packing towels (soaked in sterile isotonic saline), it was possible to bring the foetus to lie just inside that area of the uterine wall exposed by the abdominal incision. The uterus was incised for a distance of about 2 in. in a suitable intercotyledonary area, care being taken to avoid large blood vessels. The ends of the incision were identified with haemostats. The membranes, which tended to bulge through the uterine incision, were picked up with haemostats about 1½ in. apart and opened with scissors. In the sheep, the allantois does not separate the amnion and chorion completely, but usually only for about one-half the transverse circumference of the amnion. It is thus possible to enter the amniotic cavity without penetration of the allantois, provided that a chorio-amniotic and not a chorio-allantoic region is presented at the uterine incision. By making the uterine incision along the greater curvature, a chorio-amniotic area was usually presented in this series. On two occasions the margin of the allantois passed across the field but it was possible to avoid penetration of it.

Because of the delicate nature of the membranes, the edges were clamped to the margins of the uterine incision. On opening the membranes, some loss of amniotic fluid occurred. By appropriate manipulation of the foetus prior to opening the uterus it was found possible to keep this loss to less than 100 ml in most cases, and usually less than 50 ml.

An area of skin of the foetus was grasped with forceps and withdrawn above the level of the amniotic fluid while transplantation was carried out. Previous preliminary observations suggested that implantation of the grafts on the surface ("fitted" grafts) might not be successful in the foetus because of the difficulty in applying the necessary vertical pressure (Billingham and Medewar 1951). The great majority of grafts were therefore transplanted subcutaneously by the method shown in Figure 1. Two full dermal thickness specimens of skin, each about 10 by 3 mm, were removed and placed in a small blood clot at some convenient point in the operation field, care being taken to prevent dehydration of the specimen. Two small incisions (5 mm) were made about 1 in. apart and a small subcutaneous pocket effected with a probe. Into one of these was placed the autograft in the manner illustrated, and into the other the homograft from the pair mate. In the early cases, silk suture material was used to effect anchorage of the graft and to close the skin incision, but in later cases black braided nylon was adopted with a view to facilitating subsequent location. Prior to closing the membranes the area was mapped for record purposes and 300,000 units of penicillin in saline were placed in the amniotic cavity.

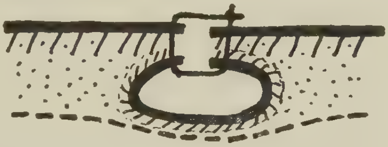

Fig. 1.—Sectional view of the method of making subcutaneous transplants.

Where fitted grafts were carried out, these were about 7 mm square and were anchored by means of a mattress suture on each of the four sides and a single suture in each corner. Contrary to expectation, they proved to be quite satisfactory as judged from subsequent observations. When the ewe was used as a homograft donor, the graft specimen was placed in aqueous penicillin (30,000 units per ml) for 5-10 min prior to grafting.

Owing to their extremely delicate nature, it was impossible to suture the membranes and the incision was therefore closed by gathering up the edges and tying off the incised area. As is indicated later, this did not prove to be an affective method of obtaining permanent closure.

Finally, the uterus was closed with a single layer of interrupted gut sutures, the abdominal wall with one or two series of gut sutures (mattress pattern), and the skin with a single layer of nylon sutures (mattress pattern). No dressings of any kind were applied to the abdominal incision, but each ewe received 10 mg progesterone and 300,000 units procaine penicillin intramuscularly immediately after the operation. Rectal temperatures were taken daily and any sheep showing a rise above 103.5°F was treated with procaine penicillin until after return to normal.

(*c*) *Histological Procedures*

All sections were fixed in formol-saline for a minimum period of 24 hr. Routine methods of dehydration of specimens were adopted and all specimens embedded in paraffin for section cutting. The blocks were cut vertically and serially, alternating 15 sections at 8 μ and 15 sections at 15 μ until exhaustion. Every fourth section only was mounted. Masson's tri-chromic stain was adopted as a standard for the majority of slides, but some slides in each case were also stained with Anderson's haematoxylin and eosin, phosphotungstic acid haematoxylin, and Giemsa.

III. Results

(*a*) *Surgical*

We are unaware of any previous attempts to make a surgical interference with the foetus in a recovery type operation as opposed to one in which the animal is killed. In fact, it seems to be commonly believed that such procedures are well-nigh impossible. This is erroneous if applied to the ewe in the last half of pregnancy. Of the 16 ewes operated on, five of which were destroyed from 9 to 22 days later, one ewe died 6 days and one aborted 11 days after operation. The remainder carried their lambs to full term. In the previous series of observations (unpublished data) in which the object was to develop the surgical technique, one of the four ewes operated on aborted 21 days later whereas the remaining three carried through to term.

Dissection of the uterus, in the five ewes on which autopsies were performed, revealed that the purse string suture applied to the membranes during the operation had become detached, leaving a hole through the chorio-amnion some 4 in. in diameter. As a result most of the amniotic fluid had escaped and only a small quantity of mucoid material remained in the amnion. Additionally, six of the nine ewes allowed to proceed to term developed herniae at the operation site, presumably due to the relatively avascular nature of the abdominal wall in this region and the increasing pressure on the ventral wall with advancing pregnancy. It seems possible that these factors, either singly or in combination, may have been responsible for the fact that five of the nine ewes had to be assisted at lambing. In all cases assisted, the lamb was normally presented but the genital tract appeared dry.

(*b*) *Grafting*

By the nature of the experiment, it was difficult to place more than one homograft and one autograft on each animal during the prenatal grafting. Only one graft of each type from each animal was therefore available for subsequent visual and histological examination. The results of the grafts have been assessed on the basis of visual and histological examinations, and are presented here in four sections:

(i) Histological examination of grafts removed from the foetuses at various post-operative intervals;

(ii) Visual examination of the operation field of the lambs at birth and during postnatal life;

(iii) Histological examination of prenatal grafts removed in postnatal life; and

(iv) Histological examination of second-set postnatal homografts.

(i) *Post-operative Histological Examination*

The sequence of histological events following skin autografts and homografts in rabbits has been described in detail (Medewar 1944) at 4-day intervals to the 24th day. The essential differences in the reaction to the two types of graft are seen in the activity and fate of the graft epithelium, the vascular reaction, and the cellular activity within the graft dermis. In *autografts* the epithelium undergoes vertical thickening and differentiation and then returns to normal thickness and morphology; the follicles regress, the hair is shed, and a new population of follicles develops. The vascular and cellular reactions of the dermis are of a relatively mild nature similar to that associated with primary healing and a mild inflammatory reaction. In *homografts*, primary healing takes place, but is followed by a series of events which probably result in the removal of the entire graft. The epithelium is undermined by a process of "blistering" and is finally shed; there is marked invasion of the dermis by capillaries which eventually stagnate and rupture, and finally, in addition to considerable fibroblast activity, there is a massive cellular invasion of the graft by lymphocytes and a few monocytes.

The current series of observations, which are based on five individuals at three post-operative stages, does not differ in any essential feature from that described by Medewar (1944) and strongly indicates the incompatibility of homografts in the foetal lamb at 110-117 days of age.

Autografts.—The single case at 9 days showed considerable increase in the vertical thickness of the epidermis, but little lateral outgrowth. There was a moderate increase in the number of dermal capillaries and a moderate oedema of the dermal collagen with new collagen being laid down: cellular elements of the graft dermis comprised mostly fibroblasts with a few lymphocytes, monocytes, and odd polymorphs in the dermal papillae (Plate 1, Fig. 1; Plate 2, Fig. 3). Generally speaking, the inflammatory reaction was negligible. Degeneration of the inner root sheath of the follicles was evident. The two cases at 15 days showed persisting thickness of the graft epithelium and its lateral outgrowth, which had progressed to the point of coalescence with the host epithelium. The follicles and their accessory structures had degenerated, leaving a mild inflammatory reaction around those fibres remaining embedded in the graft dermis. A few tortuous capillaries persisted and the graft dermis still showed some increase in the cellular population together with persisting oedema and collagen replacement. There was, however, an absence of any marked inflammatory reaction. In the 21- and 22-day cases, one had formed a complete subcutaneous cyst (Plate 3, Fig. 9), while in the other the epithelium of the graft and host had established continuity. Capillary vessels were still present, although a little reduced as compared with the 15-day cases; the cellular population of the graft dermis had practically returned to normal. Active deposition of new collagen was taking place.

Homografts.—The histological picture in the single case at 9 days was typical of the classical homograft reaction in rabbits at 8 days as described by Medewar (1944) for violent breakdown. The majority of the cells of the epidermis had lost all morphological detail and, being strongly acidophilic, stained with the light green component of Masson's stain. A few very small groups of homograft epithelial cells still showed a basophilic cytoplasm and could not be classed as dead with certainty. Additionally the epidermis had become separated from the dermis by "blistering" (Plate 1, Fig. 2). As compared with the autograft of the same animal the vascular and cellular response was strikingly greater (cf. Plate 1, Figs. 1 and 2). There was an extreme proliferation of capillaries in the dermis which had proceeded to the stage of stagnation with rupture of the endothelial lining and escape of red cells to the surrounding tissue. Not only were these vessels present in vastly greater numbers than in the autograft, but their diameter was from two to five times as great. There was a massive invasion of the dermis by lymphocytes, together with many fibroblasts. All the follicular elements were degenerate and the host epithelium was commencing to undermine the entire graft. In this 9-day case the grafts were located on the left shoulder. The prescapular lymph node draining the region was markedly enlarged, being nearly twice the diameter of the right prescapular node, and on being cut showed the characteristic glistening associated with glands draining regions in which an inflammatory reaction is taking place. Histologically the gland was apparently normal, save for considerable oedema and some congestion.

Both the 15-day cases showed a moderately violent homograft reaction, only slightly reduced from that seen in the 9-day specimen. In one case the native (host) epithelium had almost completely undermined the entire graft, whereas in the other the host epithelium had bitten deeply into the graft at about the level of the base of the follicles. The grafts showed typical dermo-epidermal blistering and disengagement. It is possible that a few homograft epithelial cells were still surviving in both cases, but they showed pronounced pathological change (Plate 2, Fig. 5). The graft dermis showed heavy lymphocyte infiltration and vascular engorgement with breakdown of the capillary endothelium and haemorrhage.

In the 21- and 22-day cases there was considerable reduction of the activity seen in the earlier homografts. The host epithelium had penetrated under the homograft from all sides, with the result that the graft was in the last stages of being shelled out (Plate 2, Fig. 6; Plate 3, Figs. 7 and 8). The fragments of necrotic graft epithelium were, at this stage, so acidophilic as to stain bright green with the tri-chromic stain. There was no trace of viable graft epithelium and both cases showed long-standing degeneration of the graft.

That the homografts had "taken" initially in all cases and become vascularized was indicated by the fact that the dermis was always extensively invaded by native mesenchyme cells, and blood capillaries which were seen in this series in varying degrees of stagnation and breakdown according to age.

(ii) *Visual Examination at Birth*

All grafts were examined, after close clipping, within 24 hr of birth. In most cases the site was readily located with the aid of the map constructed at the time of operation, and by means of the sutures where these were still present. In one case (12H75) the autograft only was located; in another (20G210) the grafts were not located until the lamb was destroyed at 5 months of age.

There was a marked difference in the nature of the autografts and homografts, irrespective of the age at which they were examined. The *autografts* (Plate 4, Fig. 11) consistently formed either a small crater about 3 mm in diameter and 4-6 mm deep with a slightly raised margin, or a subcutaneous pocket 3-5 mm in diameter and maintaining a smaller opening to the surface. In all cases the graft was producing wool fibre. The *homografts,* on the other hand, had apparently been rejected and only a small scar about 2 mm in diameter remained. It was frequently raised a little above the general skin level. Occasionally a small piece of yellow material, which was readily dislodged, was present over the graft site.

In one case in which a "fitted" autograft had been used, the graft was raised above general skin level and covered with a quantity of exfoliated material. The graft was producing normal wool fibre. One subcutaneous autograft (lamb 38S6) transplanted from the dorsal shoulder region to the anterior surface of the right metacarpus was left in situ until the lamb was 4 months old. By this time it had formed a subcutaneous 'cyst' 1.0 cm diameter and 3.0 cm long, but maintaining an opening to the surface about 3 mm diameter. The centre of the 'cyst' was filled with wool fibre and a mass of white creamy material.

(iii) *Postnatal Histology of Prenatal Grafts*

In general, postnatal histological examination confirmed the expectations from visual examination of the graft sites. As a group the autografts formed an entirely different histological series from the homografts, where the two were compared on a within-animal basis. The general picture in the autografts was one which showed a complete epithelium with a reduced follicle population, as compared with adjacent native epithelium, but accompanied by normal sebaceous glands and producing fibre (Plate 4, Fig. 11). This was so even in the youngest specimens taken at birth, when the transplants were 33 days of age. Some fibroblast activity may have remained in the dermis, but this was much reduced and disappeared rapidly. In summary, the autografts were little different from normal skin.

Scars one-quarter to one-tenth the diameter of the autografts occupied the homograft sites. Considerably increased numbers of fibroblasts and tortuous capillaries suggested a continuing reaction to, and dissolution of, parts of the graft enclosed by the host epithelium. The epithelium had returned to nearly normal thickness in the youngest specimen examined (33 days postoperative), but was devoid of follicle development. The youngest homograft site to show signs of a regenerating follicle population was examined 52 days after the original transplantation. All cases were consistent with a picture of "shelling out" and complete rejection of the homografts and subsequent repair of the graft site (Plate 3, Fig. 10). The homografts in which the ewe was used as donor were rejected in the same fashion as the foetal homografts.

As an incidental observation to the main series it is of interest to record that the regenerated follicle population in the autografts was composed entirely of secondary follicles. It has been shown (Medewar 1944, 1945) and is confirmed here, that all the follicles of an autograft degenerate soon after transplantation and are succeeded by a new follicle population developing from the basal layer of the epidermis. Two types of follicles in the skin of sheep have been demonstrated (Carter 1943); primary follicles which develop between the 30th and 80th or 85th day of prenatal life, and secondary follicles which develop thereafter. Of the four cases in this series in which it was possible to make a careful study of the regenerated follicle population, all show that this population is made up solely of secondary follicles. It is to be noted that all these grafts were carried out when secondary follicles only were being formed. It seems that the skin has lost entirely the morphogenic stimulus necessary for the production of primary follicles, even in a regenerating population.

(iv) *"Second-Set" Postnatal Homografts*

Only two pairs of lambs on which reciprocal transplants had been made during the foetal stage were available for second-set homografts during postnatal life. These comprised lamb numbers 38S1 and 38S2 (pair No. 3 of Table 1) and 38S3 and 38S4 (pair No. 4 of Table 1).

Reciprocal homograft transplants were performed between S1 and S2, under local anaesthesia, when the lambs were 13 and 12 days old respectively, i.e. 54 days after the original foetal homograft operation. Two grafts, each 1 cm by 3 mm, were reciprocally transferred from one lamb to the other, using the subcutaneous method employed earlier save that the graft was not sutured with the host skin. No control autografts were done at this stage. One homograft was removed from each animal after 5 days and the second after 8 days.

A similar technique was adopted with lambs S3 and S4, save that three reciprocal transfers were made. The first graft was removed from each animal at 4 days, the second at 9 days, and the third at 16 days from S4 and at 21 days from S3.

The major weakness of this plan is the time lag between the foetal (first-set) homografts and the postnatal (second-set) homografts. This was unavoidable because, in view of the failure to obtain closure of the amnion originally, we considered the surgical risks attached to a second penetration of the uterus and membranes to be too great.

The fate of the second-set homografts.—The main features of the breakdown of second-set homografts (Medewar 1944) are:

(*a*) The precocious invasion by, and breakdown of the primary vascular system, and as a consequence of this, the feeble cellular activity.

(*b*) The failure of the graft epidermis to undergo general vertical thickening and lateral outgrowth.

(*c*) The reduced survival time of the epidermis as compared with first-set grafts.

The current series of observations showed no appreciable variation from these criteria. In the 4- and 5-day specimens, only one of the four cases showed marked capillary invasion and engorgement. All 8- and 9-day cases showed typical second-set reactions. Blood vessels had passed through the stage of engorgement to one of complete stagnation. The lymphocytic invasion of the dermis, which is much less than that seen in the first-set grafts, had reached the point where the leucocytes had fragmented and were now dead. In only one case was there a suggestion of a "black band" (Plate 4, Fig. 13). There was no sign of hyperplasia of the graft epithelium, which gave every indication of having survived in a "vegetative" state only (Medewar 1946) (Plate 4, Fig. 13). Around the margins of the graft, proliferating epithelium of native origin had commenced to undermine either the entire graft or the graft epithelium (Plate 4, Fig. 12). The 16- and 21-day cases showed a logical progression of the above sequence of events. In each case the native epithelium had progressed to a stage of forming a complete cyst, which had shelled out the graft into the cavity of the cyst (Plate 4, Fig. 14).

The survival time of the second-set homografts was estimated as being less than 9 days, whereas that of the first-set grafts was of the order of 15-16 days. It is generally recognized that the second-dose response, which is a characteristic of actively acquired immune reactions, is best observed if the second dose is applied when the reaction to the first is at its height, or soon after. Despite the relatively long delay between first- and second-set grafts in this series, it is quite clear that a second-dose response has been observed. These results are thus in agreement with earlier observations on older animals which have shown quite clearly (Gibson and Medewar 1943; Medewar 1944) that the incompatibility normally seen following homograft operations belongs to the general class of actively acquired immune reactions, and that *innate* immunity is insufficient to account for the observed facts.

IV. Discussion

The object of these observations was to determine if skin homografts would succeed at the foetal stage in sheep. From the results obtained there seems to be no possible doubt that they do not succeed. The evidence for this comprises firstly, the contrasting histological picture of autografts and homografts seen after transplantation; secondly, the obvious macroscopic differences between the two graft types seen in lambs after birth; and thirdly, the failure to establish second-set homografts in postnatal life, using the same donor and recipients as were used for the prenatal transplants. Within the present series, age had no obvious effect on the reaction.

There is no doubt that the foetal reaction to the skin homografts in this series is one of active rejection by the foetus; it is not merely a failure of the graft to "take" due to inadequate vascularization. That the reaction is of foetal and not maternal origin is indicated by the fact that, in both cases in which maternal skin was grafted to the foetus, the homografts were rejected. It must be concluded therefore that the foetus is able to make an immune response. This appears to be a contradiction of such statements as "foetal mammals and chick embryos are incapable of producing antibody" (Burnet and Fenner 1949, p. 102) and "the embryo is immunologically inactive" (Anderson *et al.* 1951).

We are unaware of any previous work which takes the form of a direct study of the ability or otherwise of foetal mammals to produce an immune response. The fact that the foetal lamb is born with no circulating antibody following the introduction of bacterial antigens into the ewe (Mason, Dalling, and Gordon 1930) cannot be considered evidence of the failure of the foetus to produce antibody. There is considerable evidence that such antigens do not cross the placental barrier in sheep, thus the lamb has no opportunity to produce a response.

The main studies of the ability of embryos to produce antibodies have been carried out in the developing chick (Grasset 1929; Burnet 1941). Grasset has shown that no immunity to diphtheria toxin was developed in the chick following injection of the egg with the corresponding toxoid on the 7-18th day of incubation. Similarly, Burnet showed that influenza virus, which is a potent antigen in adult fowls, failed to stimulate antibody production in the developing chick when inoculated on the twelfth day of incubation.

Two possibilities are suggested to account for the discrepancy between the results of observations recorded in this paper and current concepts of the immunological behaviour of embryos. Firstly, it may be that the foetal lamb is "abnormal" in the sense that it acquires an antibody-producing mechanism at an early age. In an immunological sense, the new-born lamb does appear to be more mature than, for example, the new-born mouse. The latter has neither red-cell antigens nor the capacity to produce antibody at birth. On the other hand, Ycas (1949) has shown that the lamb at birth possesses nine red-cell antigens capable of detection with prepared antisera, although two further antigens and the corresponding naturally occurring iso-antibodies are not present until 2-3 weeks of age. Secondly, it is possible that the foetus is unable to respond immunologically to antigens which have a determinant pattern for antibody production widely different from its own "marker" pattern, using the term "marker" in the concept of Burnet and Fenner (1949). If this were so it would account for the failure to observe antibody production in chick embryos in response to the bacterial and viral antigens. At the same time the hypothesis allows for the development of an immune response, as recorded here, where the antigen is not of greatly different genetic makeup from the recipient. This requires that the foetus has the ability to respond to "individuality differentials" (Loeb 1945) of small degree (as occurs in homografts), but not to those of extreme differences (heterografts and bacterial and viral antigens). Although there is little direct evidence to support such a hypothesis, it may be recalled that the cellular reaction varies according to the degree of genetic difference, or "individuality differential," of donor and recipient. Loeb (1945) has drawn attention to the fact that the reaction to homografts is essentially one of lymphocytes, fibroblasts, and a few monocytes, whereas the reaction to antigens of wider differentials is largely of polymorph leucocytes. The predominant part played by lymphocytes in the homograft reaction has been amply demonstrated by Medewar (1944, 1945) and has been observed consistently in these observations.

In dizygotic cattle twins, each member receives cellular components from its opposite number at a very early age, whereas in these observations the earliest homografts did not take place until the 80th day of prenatal life. There must be a zero point in the ability of the embryos to recognize an antigen as "non-self." This point must be between fertilization and the 80th day of prenatal life in the sheep.

V. Acknowledgments

The preliminary observations to this series were carried out while the authors were working at the University of Cambridge, England, under studentships of the C.S.I.R.O.

It is a great pleasure to record the encouragement and advice, freely given throughout these observations, of Professor P. B. Medewar of University College, London, and particularly to acknowledge his guidance in the interpretation of the histological material. Our thanks are also due to Mr. F. J. Fraser for his excellent histological preparations and to Dr. R. A. Barter and Mr. Keith Phillips for assistance with the photomicrography.

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EXPLANATION OF PLATES 1-4

PLATE 1

Fig. 1.—Autograft, 9 days, showing vertical thickening of the epithelium and early stages of follicle degeneration. Masson's tri-chromic.

Fig. 2.—Homograft, 9 days, from same animal as autograft in Figure 1. Note dermo-epidermal "blistering" and disengagement of the epidermis and vascular and cellular reaction in graft dermis. Masson's tri-chromic.

PLATE 2

Fig. 3.—Autograft, 9 days, Masson's tri-chromic.

Fig. 4.—Homograft, 9 days, Masson's tri-chromic.

Fig. 5.—Homograft, 15 days, showing "blistering" of the epithelium, which is not certifiably dead, but shows pronounced pathological changes. Note lymphocyte invasion of dermis. Masson's.

Fig. 6.—Homograft (fitted), 22 days. The graft has been almost completely undermined by native epithelium growing in from each side. Note persisting reaction in graft dermis. Masson's.

PLATE 3

Fig. 7.—Homograft, 21 days, showing the last stage of the undermining process by the native epithelium. H & E.

Fig. 8.—Homograft, 21 days, in the last stages of being "shelled-out" by native epithelium. The dark mass of the centre comprises partly new cuticle formed by the new epithelium and partly the necrotic graft. Masson's.

Fig. 9.—Autograft, 21 days. The graft has become completely encysted at this stage, the centre of the cyst being filled with wool fibres and a mass of exfoliated cuticle. H & E.

Fig. 10.—Homograft, 33 days. The graft has been completely undermined by native epithelium to form a cyst similar to that in Figure 9, except that the centre of the cyst is now filled with the necrotic remnants of the graft together with fragmented mesenchyme cells. Masson's.

PLATE 4

Fig. 11.—Autograft, 82 days, showing the 'pit-like' form and the development of follicles. Masson's.

Fig. 12.—Second-set homograft, 8 days, showing the development of native epithelium, which is commencing to undermine the entire graft. H & E.

Fig. 13.—Second-set homograft, 8 days, showing cellular invasion of the graft dermis, reminiscent of the "black band" observed by Medewar only in first-set grafts. Note the "vegetative" condition of the graft epithelium. H & E.

Fig. 14.—Second-set homograft, 21 days. The graft has been completely "shelled-out" and lies in the cavity of the cyst formed by native epithelium. H & E.

SKIN TRANSPLANTATION IN FOETAL LAMBS

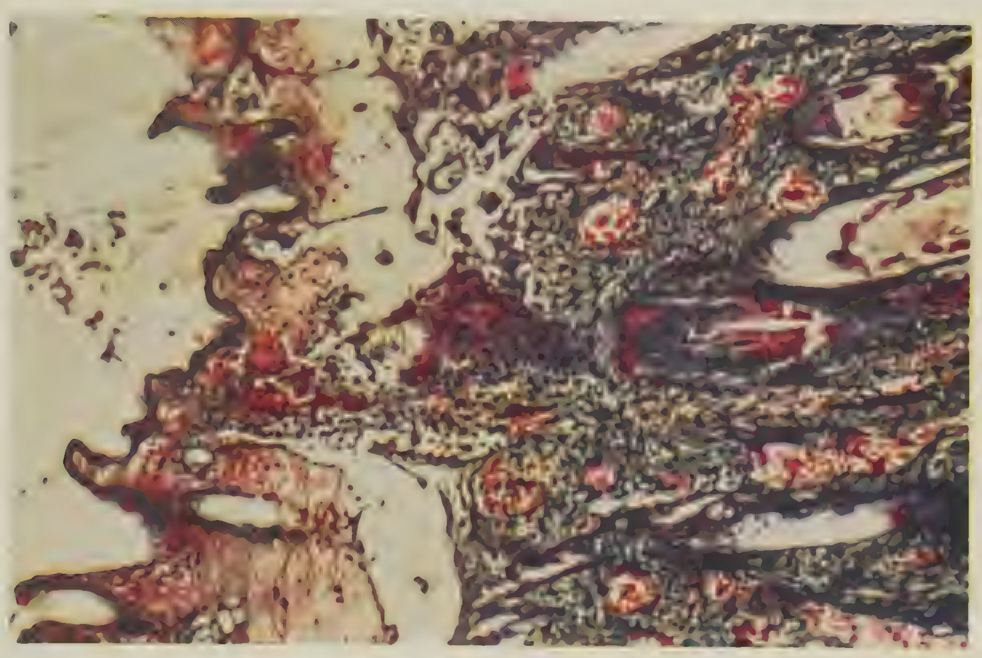


Fig. 2

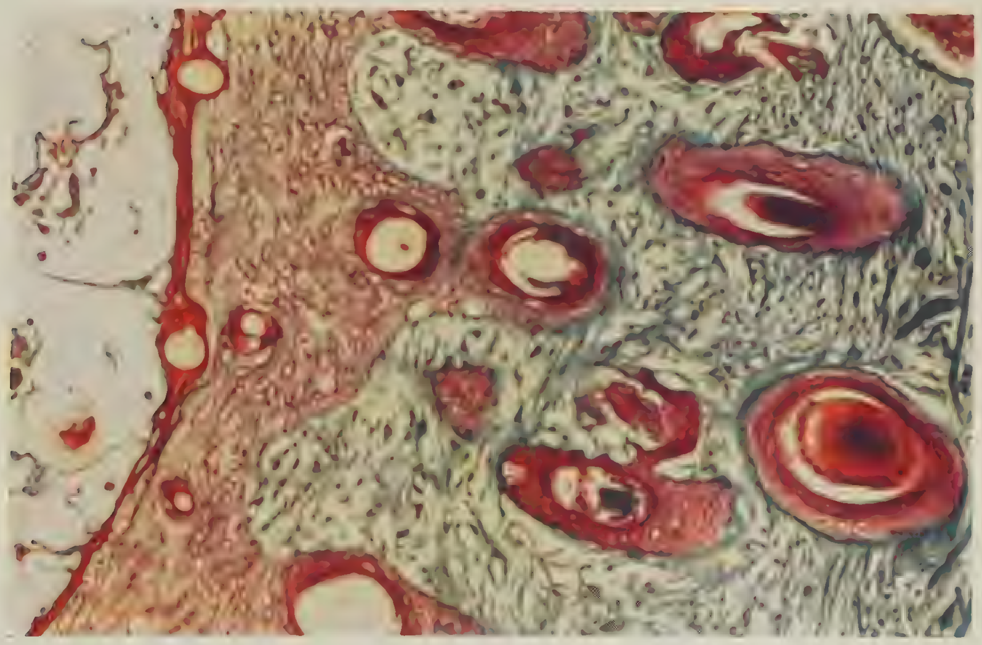
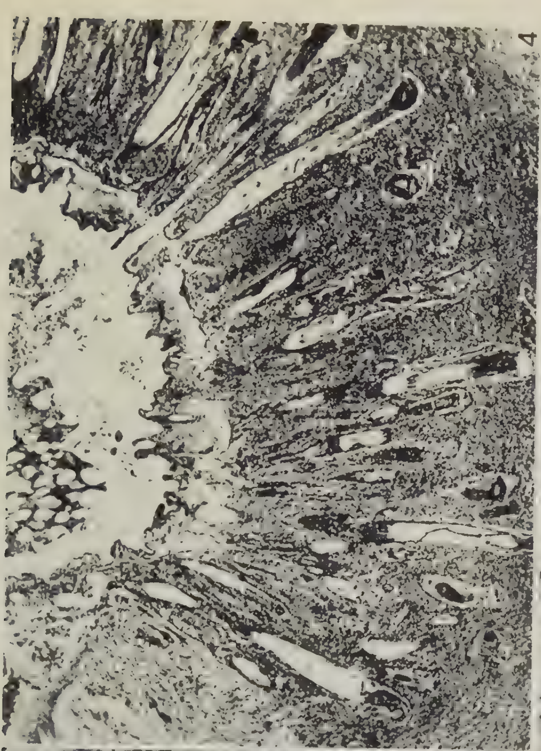


Fig. 1

SKIN TRANSPLANTATION IN FOETAL LAMBS



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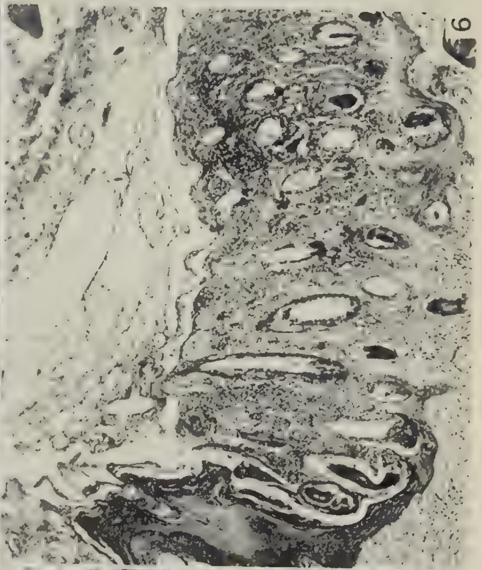

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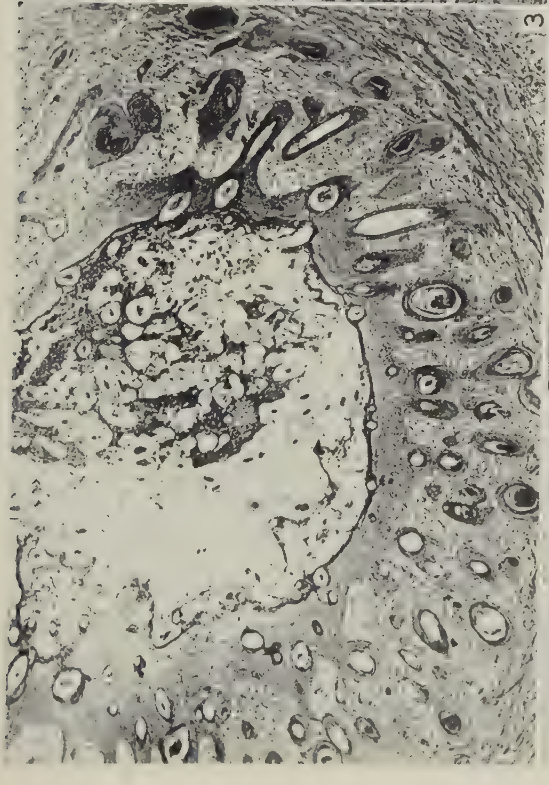

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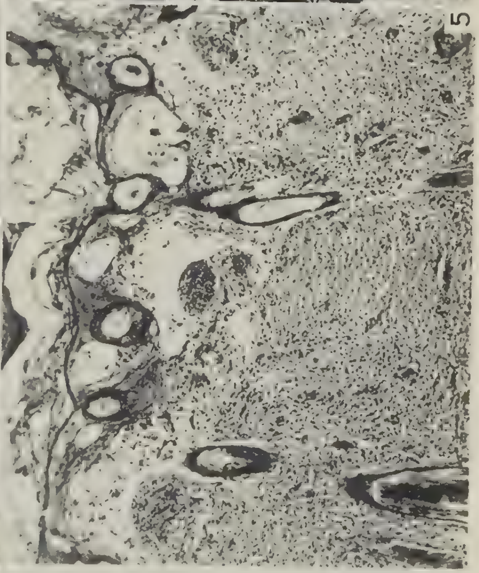

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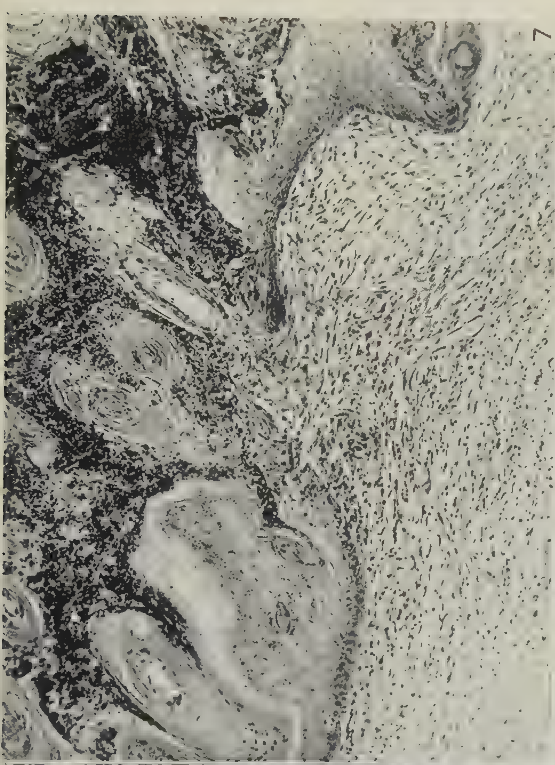
SKIN TRANSPLANTATION IN FOETAL LAMBS



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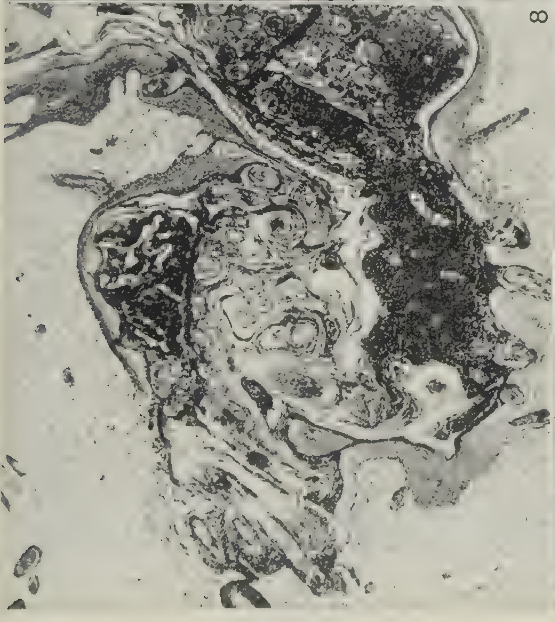

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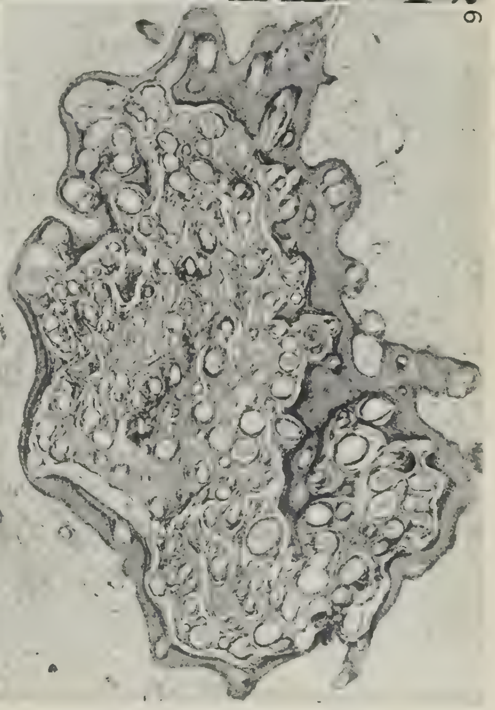

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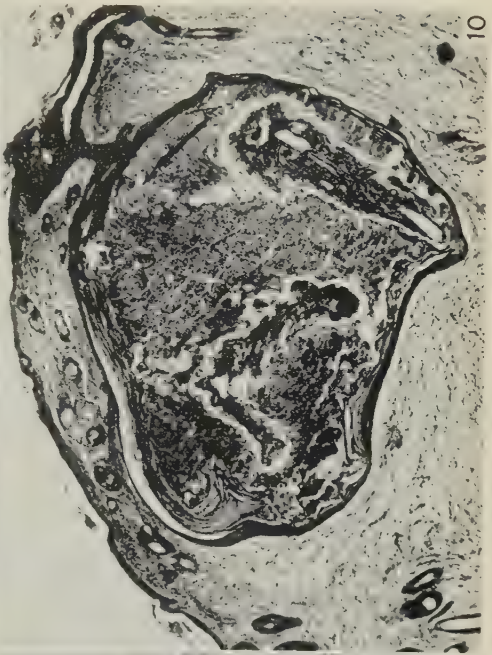

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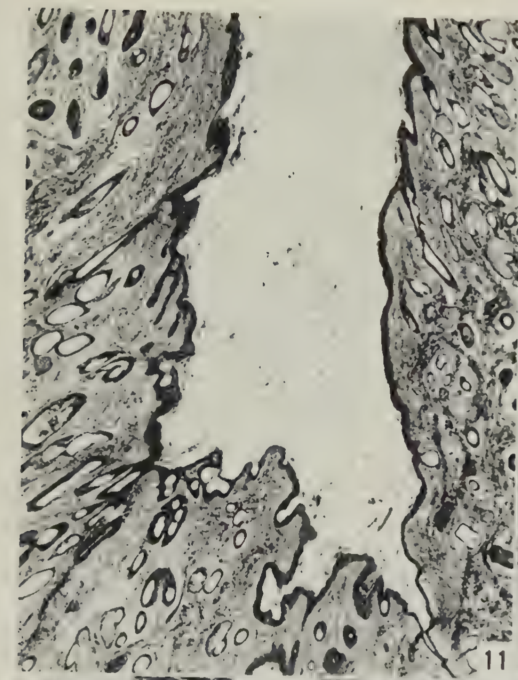
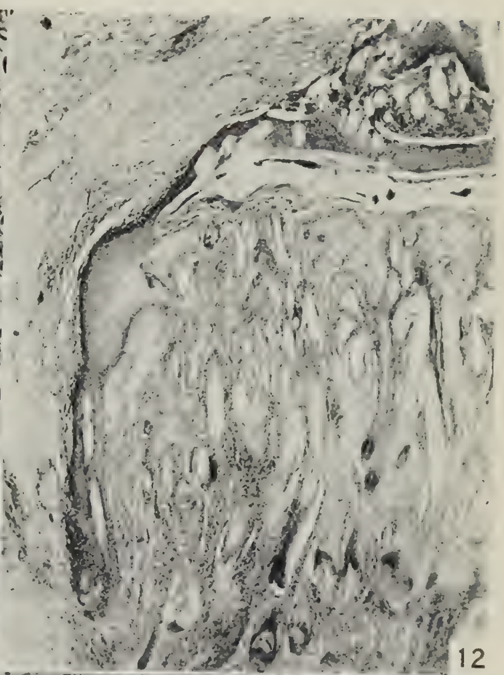
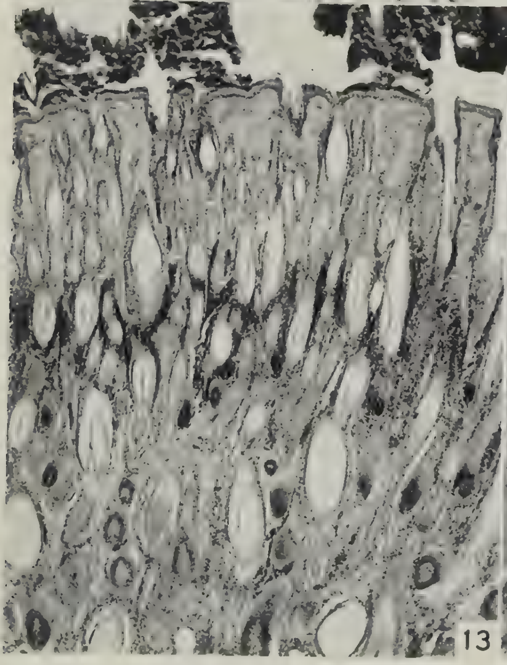
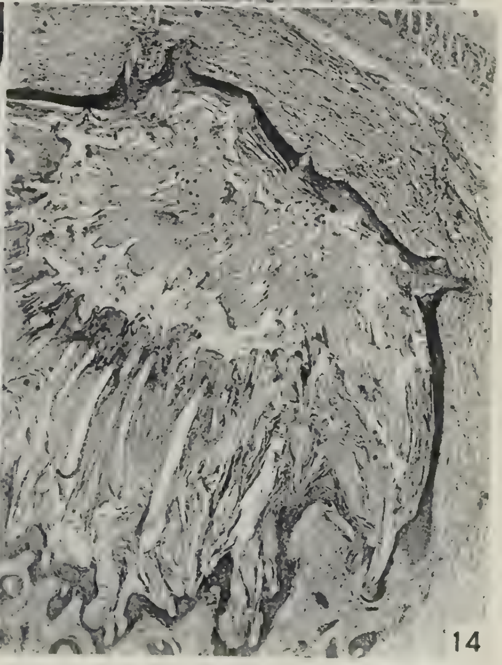
SKIN TRANSPLANTATION IN FOETAL LAMBS









CORRIGENDUM

VOLUME 6, NUMBER 1

Page 40, Table 12: The following footnote was omitted: The data listed in columns 4-9 inclusive were obtained by Mr. D. E. Bland, Division of Forest Products, C.S.I.R.O.

WATER RELATIONS OF *STAPHYLOCOCCUS AUREUS* AT 30°C

By W. J. Scott*

[*Manuscript received April* 30, 1953]

Summary

Fourteen food-poisoning strains of *Staphylococcus aureus* have been grown in various media of known water activity at 30°C. Aerobic growth was observed at water activities between 0.999 and 0.86. The rate of growth and the yield of cells were both reduced substantially when the water activity was less than *c.* 0.94. The lower limits for growth in dried meat, dried milk, and dried soup were similar to those in liquid media. Aerobic growth proceeded at slightly lower water activities than anaerobic growth. All cells were capable of forming colonies on agar media with water activities as low as 0.92. The 14 strains proved to be a homogeneous group with similar water requirements.

I. INTRODUCTION

There is general agreement that the influence of the availability of water on the growth of microorganisms is most appropriately considered in terms of the relative humidity. Most of the experimental work has been done with moulds, especially with those which are able to grow at comparatively low relative humidities. Some of the experimental requirements have been stated by Tomkins (1929). The most important of these is that the water vapour pressure of the substrate should be in equilibrium with that of the atmosphere in which it is stored. It is only by observing this condition that it is possible to ensure uniform distribution of water through the substrate and a constant water content throughout the duration of an experiment. The experiments with moulds are usually carried out on thin films of solid media in equilibrium with sulphuric acid solutions of known composition. Walter (1924), who was one of the first to employ this technique, made some observations with bacteria. He found that none of the bacteria tested could grow at a relative humidity of less than 96 per cent., whereas several moulds grew at much lower humidities. Scott (1936), working with thin slices of beef muscle equilibrated with sulphuric acid solutions, found two strains of *Achromobacter* to have a lower limit for growth of approx. 96 per cent., and two strains of *Pseudomonas* to have a lower limit greater than 98 per cent. These experiments were done at −1°C. No other studies of bacteria growing under conditions of controlled humidity appear to have been made although many workers have studied the effects of the concentration of various solutes on the growth of bacteria. Many of these observations have been made in media for which useful approximate calculations of the equilibrium humidities can be made.

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Observations on staphylococci have shown that they generally tolerate rather higher solute concentrations than most other bacteria, and this has formed the basis of media used for enrichment and isolation of staphylococci as proposed by Chapman (1946). Segalove and Dack (1951) reported the growth of various food-poisoning bacteria on dried meat at different water contents. They found that a food-poisoning strain of S. *aureus* grew at rather lower water contents than the other organisms tested. The equilibrium humidity corresponding to the limiting water contents was, however, not given. The present paper reports the results obtained when the growth of 14 food-poisoning strains of S. *aureus* was studied in several media in which the availability of the water was controlled.

II. Methods

As the methods used for controlling the availability of water are of paramount importance and are not recorded fully elsewhere, the essential features are given in some detail. Firstly, the equilibrium humidity of an aqueous solution is the relative humidity of an atmosphere which neither gains water from nor loses water to the solution. In such a system at equilibrium the water vapour pressure of the solution is the same as in the atmosphere above it. The water vapour pressure of the solution expressed as a fraction of the vapour pressure of pure water at the same temperature (or the equilibrium humidity expressed as a fraction) is numerically equal to the activity of the water (a_w) in the solution. It will be convenient henceforth to use this quantity as a measure of the availability of water in the medium; its relation to osmotic pressure and other properties of the solution are discussed in the various textbooks on physical chemistry.

In studying the growth of bacteria on a liquid medium such as nutrient broth at various levels of a_w it is convenient to construct the vapour pressure isotherm of the medium at the appropriate temperature. This curve shows the relation between water contents and a_w at the particular temperature. Although strictly applicable only at one temperature, the isotherm is not greatly affected by temperature and in practice it is reasonably safe to assume that the isotherm will be virtually unchanged at other temperatures within 5°C.

Table 1 shows the equilibrium water contents for two lots of brain-heart infusion (B.H.) medium for various round values of a_w at 25°C. Determinations were made at approximately 12 points between a_w's of 0.75 and 0.99 by the isopiestic technique of Robinson and Sinclair (1934). Solutions, in lots of about 2 ml, were equilibrated in gold-plated silver dishes made to fit D40 standard taper glass lids. Four such dishes were placed in a copper block weighing approximately 1.5 kg. The copper block was placed in an evacuated desiccator, which was submerged in a water-bath at 25 ± 0.01°C and continuously rotated in a plane about 12° from horizontal at 40 r.p.m. The reference solution was sodium chloride (Robinson 1945). After equilibrium was reached the composition of the solutions was obtained from the dry weight after 20-24 hr at 105°C.

Determinations made by this method fell on a smooth curve, and it will be seen from Table 1 that there is close agreement between the two batches prepared according to the same formula. At a_w's greater than 0.99 the solutions are dilute and departures from Raoult's law are small. If, therefore, the equilibrium water contents are known at an a_w close to 0.99 it is permissible to calculate the water contents corresponding to any a_w above 0.99 by assuming that a_w is proportional to the mole fraction of water. The assumption is valid for un-ionized and completely ionized solutes, and also for partially ionized solutes provided that the degree of ionization does not change appreciably with further dilution. It is not necessary to know the molecular weights of the solutes concerned, as for any given mixture of solutes the mole fraction of the solvent is inversely proportional to solute concentration on a weight basis. It is convenient to express water contents in terms of the dry weight of solute, as values so expressed are directly proportional to the amount of water in any given system. There is the additional advantage that such values are inversely proportional to concentration per unit weight of water and molality (m). When the vapour pressure isotherm for any particular medium has been determined, it is possible to make media with water contents corresponding to any desired value of a_w. It is to be noted, however, that any reduction of water contents inevitably introduces an associated increase in the concentration of nutrients. If therefore it is desired to ensure that the reactions of the bacteria are related to the availability of water it is necessary to extend the observations to other media with a different relation between nutrient concentration and a_w. By constructing isotherms for other media it is possible to compare the reactions of the organisms at the same a_w in the presence of different types of nutrients.

TABLE 1

EQUILIBRIUM WATER CONTENTS OF TWO LOTS OF BRAIN-HEART INFUSION MEDIUM AT VARIOUS ROUND VALUES OF a_w AT 25°C

a_w	Dehydrated Infusion (Difco) (H_2O % dry wt.)	Infusion Prepared in Laboratory and 40 l. Dried from Frozen State (H_2O % dry wt.)
0·99	1990	2080
0·98	950	1030
0·97	620	650
0·96	472	490
0·94	312	320
0·92	245	230
0·90	200	185
0·88	165	150
0·86	135	125
0·84	—	102
0·80	—	82
0·75	—	67

It is also possible to vary a_w by adding non-nutrient solutes in varying concentration to a basal medium in which the concentration of nutrients is maintained at a constant level. This may be done very simply by adding solutes such as sodium chloride or sucrose to nutrient broth. Such experiments are frequently performed, but the results are usually not reported in terms of a_w but only in terms of solute concentration. Osmotic coefficients for a number of electrolytes and some non-electrolytes have been published in recent years (Stokes 1948; Robinson and Stokes 1949) and it is possible to calculate the a_w of solutions over a wide range of concentrations at 25°C. It is also possible to deduce with considerable precision the a_w of solutions containing mixtures of various solutes (Robinson and Stokes 1945) when these do not react to form insoluble compounds or complex salts. This procedure has been used in these experiments using a basal medium of mineral salts, casamino acids, yeast extract, and casitone (C.Y.C.), which contains all the nutrients required by *Staphylococcus aureus.* The composition of this medium is given in Table 2 together with the contribution of each ingredient to the vapour pressure lowering. The contributions of the complex ingredients are deduced from freezing-point determinations made on solutions of known composition. In dilute solutions of low ionic strength simple addition of the various values of $1 - a_w$ is permissible, and it is concluded that the medium has an a_w close to 0.999. The ionic strength (μ) is of the order of 0.04-0.05, the actual value being dependent on the composition of the complex ingredients and, as phosphate is a major constituent, on the pH.

TABLE 2

COMPOSITION OF BASAL C.Y.C. MEDIUM WITH AN a_w OF 0.999 AT 25°C

	m	g/1000 g H_2O	Calculated $(1-a_w)$
Na_2HPO_4	0·01	1·42	0·00033
KH_2PO_4	0·002	0·27	0·00007
$MgSO_4$	0·001	0·12	0·00002
NH_4NO_3	0·005	0·40	0·00016
Glucose	0·01	1·80	0·00018
Casamino acids		0·60	0·00013
Yeast extract		0·10	0·00002
Casitone		0·20	0·00002
Total		4·91	0·00093

Calculated a_w of medium is therefore 0·99907, or *c.* 0·999.

This C.Y.C. medium was adjusted to lower levels of a_w with various solutes. Most of the experiments were made when the adjustment was made with a mixture of NaCl, KCl, and Na_2SO_4 in the ratio of 5:3:2. The method for calculating the a_w of the mixture was adapted from the "second approximation" given by Robinson and Stokes (1945). This method depends on the assumption that the relative molal lowering of the vapour pressure, $(1-a_w)/\mathrm{m}$, for each salt is a function of the total ionic strength of the mixture. The calculations are made in two stages. Firstly, the value of $(1-a_w)/\mathrm{m}$ is plotted against μ for each salt separately. The a_w of the mixture is then calculated for several round values of μ and a second curve is constructed relating a_w and μ for the particular salt mixture. From this curve, or by linear interpolation, the value of μ corresponding to any desired value of a_w may be obtained, and from the ionic strength so obtained the molalities of the individual salts are determined.

In determining the quantities of salt to be added to a given amount of C.Y.C. basal medium the calculation was simplified by neglecting the small contribution to the final ionic strength made by the basal medium. The final adjustment is then made by simple addition of $1-a_w$ for the basal medium and $1-a_w$ for the electrolyte mixture. For the particular mixture specified the concentrations of salts required to give various round values of a_w in C.Y.C. medium are given in Table 3. The composition of other mixtures used has been calculated in a similar way. Robinson and Stokes (1945) found that the calculated vapour pressure lowering of mixtures was within about 1 per cent. of the actual value, for water activities down to about 0.8. A check by the isopiestic technique showed agreement within this limit for the mixture in Table 3 with a calculated a_w of 0.920.

TABLE 3

MOLAL CONCENTRATIONS OF SALTS REQUIRED FOR ADJUSTING C.Y.C. MEDIUM OF a_w 0.999 TO VARIOUS VALUES OF a_w AT 25°C WITH A 5 : 3 : 2 MIXTURE OF NaCl, KCl, AND Na_2SO_4

Final a_w in Medium	$(1-a_w)$ of Salt Mixture	NaCl (m)	KCl (m)	Na_2SO_4 (m)
0·995	0·004	0·0575	0·0345	0·0230
0·990	0·009	0·1293	0·0776	0·0517
0·980	0·019	0·2789	0·1673	0·1116
0·960	0·039	0·5805	0·3483	0·2322
0·940	0·059	0·869	0·521	0·348
0·920	0·079	1·149	0·690	0·460
0·900	0·099	1·418	0·851	0·567
0·880	0·119	1·663	0·998	0·665
0·860	0·139	1·921	1·153	0·768

Media were made by adding the appropriate quantities of anhydrous salts to aliquots of basal medium containing a known weight of water. After the salts were dissolved the mixture was sterilized by autoclaving, with precautions against evaporation. Any loss of weight was restored with sterile distilled water. All media were made on the basis of dry ingredients, and corrections for water in substances such as meat extract were applied when anhydrous materials were not used. The a_w of a particular mixture was checked by determination of the dry weight before commencing an experiment. During an experiment precautions were taken to ensure that the a_w did not change by more than 0.001. The precautions necessary depended on the duration of the experiment, the weight of water in the system, and the a_w. The latter is important because, at low values of a_w, a given quantity of water causes a greater change in a_w than in more dilute solutions.

Table 4

SOURCES AND PHAGE PATTERNS OF THE 14 STRAINS OF *STAPHYLOCOCCUS AUREUS*

Source	Strain No.	Phage Pattern
Central Public Health Laboratory, Colindale, London England (Dr. R. E. O. Williams)	49/1255	6/7/42B/47/47B/47C/53/54
	49/1284	6/47
	49/1974	42D
	50/225	6/7/42B/47/47B/47C/53/54
	50/3002	7
	50/3147	6/42B/47/53/54
	50/3185	6/42B/47
American Meat Institute, Chicago, U.S.A. (Dr. C. F. Niven, Jr.)	F2B	47/53
	K57	47
	S6	47/54
	S12	3A/3B/51
	S209	47
	210	47
Fairfax Institute of Pathology, Sydney, Australia (Dr. Phyllis Rountree)	R4730	7/42B/47B/53

Aerobic growth at water activities of 0.90 and above was measured nephelometrically in 15-mm Pyrex T tubes containing 10 ml of medium and rocked continuously in a water-bath at 30°C ± 0.05. Anaerobic growth rates were measured in sealed Pyrex tubes at the same temperature. The method of sealing the tubes and the characteristics of the nephelometer have been described by Ohye and Scott (1953). Each μA of the nephelometer was equivalent to approx. 3.0×10^5 cells/ml in C.Y.C. medium at several a_w's between 0.90 and 0.999. The corresponding dry weight of cells was 4.3×10^{-2} μg at 0.999 a_w. The correct determination of dry weight of cells at low values of a_w is being studied further. Growth in opaque media was followed by viable counts using the technique of Miles and Misra (1938) and counting colonies after 48 hr at 30°C on brain-heart agar. Dilutions were made in 0.9 per cent. saline. The use of isotonic diluents was not necessary. Growth at water activities of 0.88 and below was studied visually or by viable or direct counts. The observations were continued over 30 days at 30 ± 0.5°C. Cultures of about 20 ml were placed in 100-ml screw-capped bottles sealed with paraffin to reduce evaporation.

The lower limits of a_w permitting growth were studied also in three foods. For these the vapour pressure isotherms were determined at 25°C using the isopiestic technique. Aqueous suspensions were spread uniformly as a thin film on the bottom of the silver dishes and sorption isotherms determined between 0.75 and 0.98 a_w. For this purpose it was convenient to use as reference solutes the saturated solutions listed by Stokes and Robinson (1949). When being prepared for growth studies aliquots of approx. 10 g of dry food were brought to the appropriate water contents by the cautious addition of the required weight of distilled water, the mass being continuously agitated with approx. 20 g of glass beads in a 100-ml screw-capped bottle. This ensured the even distribution of water through the food. Sterilization for 30 min at 100°C was adequate. After inoculation the bottles were shaken to distribute the inoculum, and initial samples taken for viable counts and estimate of water contents. The bottles were incubated at 30 ± 0.5C within desiccators over sodium chloride solutions of the same a_w. This precaution was found to be necessary as the total amount of water in some of the bottles was only about 1 g and evaporation of only 5 mg/day would have been serious over a period of 30 days. Viable counts and water contents were determined at intervals.

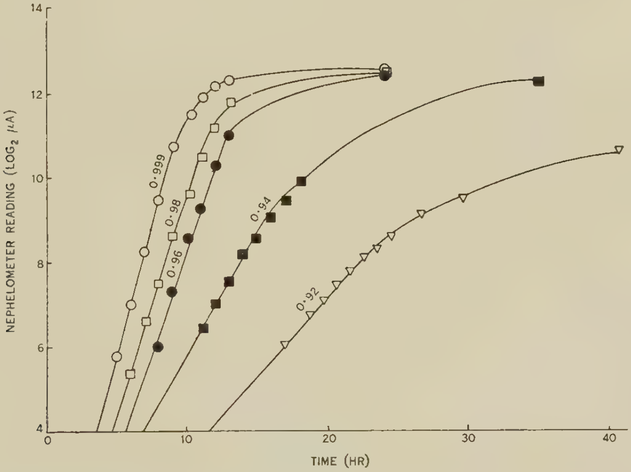

Fig. 1.—Growth curves for *S. aureus* strain 50/3002 on C.Y.C. medium adjusted to various levels of a_w with salts mixture listed in Table 3.

All cultures were inoculated with 0.02 ml of a saline suspension prepared from an overnight culture in nutrient broth. For the liquid media the inoculum gave an initial count of about 10^5 cells/ml and for the foods about 10^6 cells/g.

Experiments have been made with 14 cultures of *Staphylococcus aureus,* the organisms being typical of the species as described by Shaw, Stitt, and Cowan (1951). All strains had either been implicated in outbreaks of food poisoning or had been shown to produce enterotoxin. The phage patterns of the different strains were kindly determined by Dr. Phyllis Rountree using the method described by Williams and Rippon (1952). The phage patterns and the origins of the various cultures are shown in Table 4. With the exception of strain S.12 all strains belong to the 6/47 group of Williams and Rippon, this group including almost all the food-poisoning strains isolated in England.

III. Results

(*a*) *Growth in C.Y.C. Medium*

Some typical results for one strain grown in C.Y.C. medium are shown in Figure 1. The various levels of a_w were obtained by adding the appropriate quantities of the salt mixture given in Table 3. Reduction of the a_w below 0.99 caused a progressive decrease in both the rate of growth and the maximum density of the culture. The results with other strains were very similar and when the results for the different strains were compared no consistent differences between strains were detected. Figure 2 shows the average results for all strains on C.Y.C. medium. The greatest rates of growth were found at a_w's of 0.995 and 0.99, the rates at these a_w's being significantly greater ($P < 0.01$) than the rate at an a_w of 0.999. As the a_w decreased from 0.99 to 0.90 there was a progressive reduction in the rate of growth to a level less than 10 per cent. of the maximum rate. The yield of cells also decreased progressively over this range.

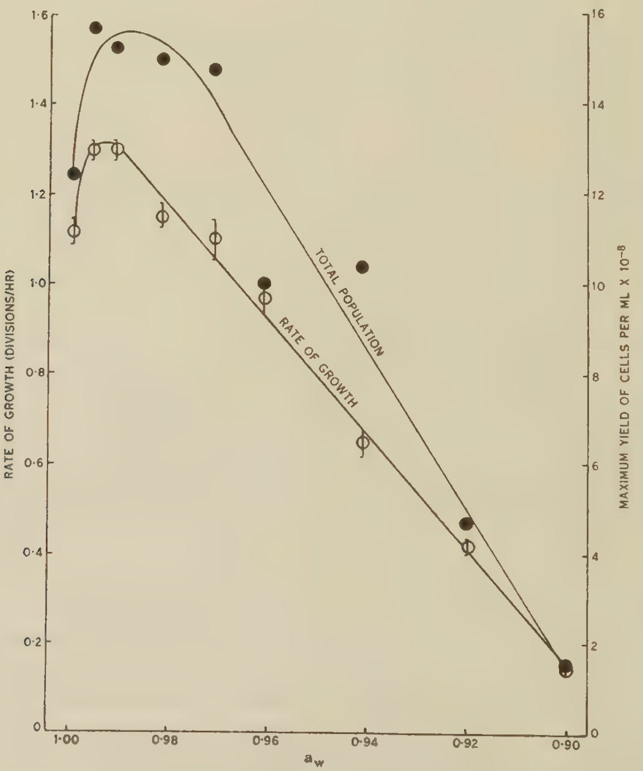

Fig. 2.—Relation between mean rate of growth, maximum yield of cells, and a_w for 14 strains of *S. aureus* grown in C.Y.C. medium with added salts.

(*b*) *Growth in B.H. Medium*

When the B.H. medium dried from the frozen state was adjusted to various a_w's by preparing media with the water contents shown in Table 1, the results shown in Figure 3 were obtained. At the higher a_w's the medium was sufficiently transparent for rates of growth to be determined nephelometrically and by viable counts, but in the more concentrated media light-scattering measurements were not possible. In this medium somewhat greater rates of growth were realized at high a_w's, but as the a_w fell below 0.94 the rates were very similar to those shown in Figure 2 for the C.Y.C. medium with added electrolytes. It may be noted that the yield of cells/ml increased with increasing concentration of nutrients as the a_w was decreased from 0.995 to 0.98 and 0.96, but at lower a_w's the yield of cells tended to decline. The lag period in B.H. medium (not shown in Fig. 3) increased from *c.* 1 hr at 0.995 a_w to 3-6 hr at 0.96 a_w, and at lower a_w's further increased to over 3 days at 0.90 a_w. In this medium the lag periods at the lower a_w's were much longer than on C.Y.C. medium, in which the deduced lag was less than 24 hr at 0.90 a_w. Owing to these considerable differences between media it is not possible to make a useful quantitative statement about the length of the lag period in relation to a_w.

TABLE 5

MINIMUM a_w FOR GROWTH OF *S. AUREUS* IN VARIOUS LIQUID MEDIA AT 30°C

+ = Growth and — = no growth, within 30 days. All cultures tested reacted similarly except where fraction in brackets indicates a smaller number

Basal Medium	Solutes Added	Number of Strains Tested	a_w		
			0·88	0·86	0·84
C.Y.C.	Salts mixture*	14	+($^{12}/_{14}$)	—	—§
Nutrient broth	Salts mixture*	13	+	+($^{9}/_{13}$)	—§
Nutrient broth	Sucrose	6	+	—	—
Nutrient broth	3·44m Sucrose + salts mixture*	6	+	+($^{5}/_{6}$)	—
Nutrient broth	4·07m Glucose + salts mixture*	6	+	—	—
Nutrient broth	0·11m Glucose + salts mixture*	1	+	—	—
Brain-heart infusion	None†	1	—‡	—	—

* Details in Table 3. † Water contents in Table 1.
‡ Growth at 0·90a_w. § Saturated solution, therefore a_w approximate only.

(*c*) *Minimum* a_w*'s for Growth*

The lower limits for growth have been studied in several liquid media adjusted to different a_w's with various solutes. The results are summarized in Table 5. Slow growth has been observed down to 0.86 a_w, but not at 0.84 a_w. There is little evidence of variation between strains and, in the media used, the results are similar for the various solutes used to control a_w.

(*d*) *Growth in Foods*

The lower limits of a_w at which growth is possible have also been studied in three foods, dried milk, dried mutton, and a commercial dried soup mixture.

The equilibrium water contents for these foods at several round values of a_w are shown in Table 6. The figures have been taken from the respective vapour pressure isotherms at 25°C. When these foods were inoculated and incubated at 30°C the results shown in Figure 4 were obtained. Slow growth occurred in all foods at 0.88 a_w and in the milk and soup at 0.86 a_w. The results show satisfactory agreement with those obtained in liquid media (Table 5), and demonstrate that it is a_w rather than water content which determines whether or not a substrate is sufficiently moist to support growth of *S. aureus*.

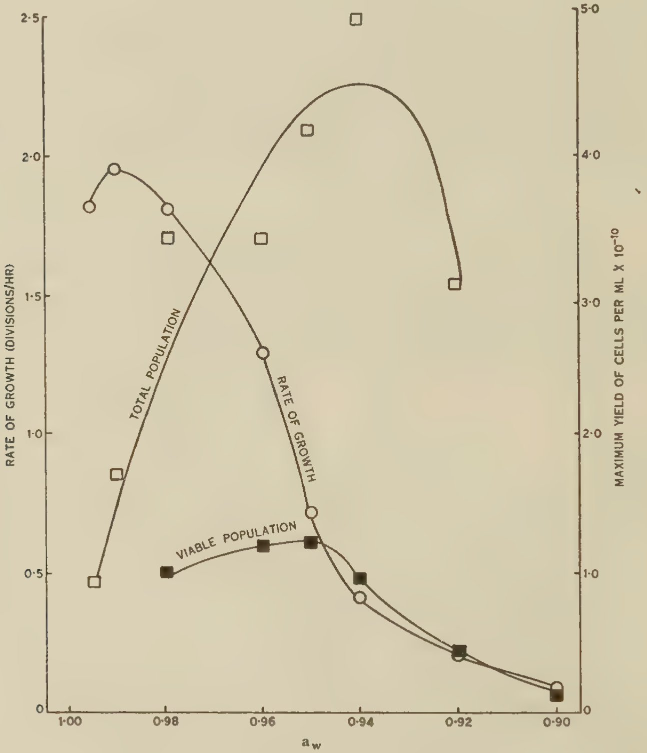

Fig. 3.—Relation between rate of growth, total and viable populations, and a_w for *S. aureus* strain 49/1974 growing on brain heart infusion.

(*e*) *Comparison of Aerobic and Anaerobic Growth*

All the foregoing experiments were carried out under aerobic conditions, and some comparisons have therefore been made of aerobic and anaerobic growth at various a_w's. The results are shown in Figure 5. The aerobic rates were similar to those found in B.H. medium, being 1.85, 1.18, and 0.45 divisions/hr at a_w's of 0.995, 0.96, and 0.92 respectively. The corresponding rates under anaerobic conditions were 1.37, 0.68, and 0.09. At 0.88 a_w, growth occurred aerobically but not anaerobically within 4 wk at 30°C. Plots of the anaerobic rate and of the ratio of the anaerobic to the aerobic rates against

a_w both indicate that anaerobic growth is unlikely at an a_w of 0.90 in this medium. The yield of cells from anaerobic growth was less than 10 per cent. of the aerobic yield at all a_w's.

(*f*) *Effect of* a_w *on the Plate Count*

The relation between the plate count and a_w was studied in nutrient agar adjusted with mixed electrolytes to various desired levels of a_w down to 0.90. The basal medium was obtained by adding 1 per cent. of agar to nutrient broth. The water activity was reduced by less than 0.001 a_w by the addition of this amount of agar. The results are shown in Table 7. χ^2 tests on the counts of the triplicate plates showed that all the counts down to 0.92 a_w were homogeneous for the cells grown in nutrient broth. For the cells grown in C.Y.C. medium with mixed electrolytes at 0.96 a_w, there was a significant decrease in the plate count at a_w's of 0.94 and 0.92. With both types of inoculum no colonies were detected at 0.90 a_w after 11 days at 30°C. Two conclusions of importance emerge. The first is that all the cells in the population grown in nutrient broth were able to grow at a_w's at least as low as 0.92. The second is that cells grown at 0.96 a_w had no greater capacity to form colonies at low a_w's than cells grown at 0.999 a_w.

TABLE 6

EQUILIBRIUM WATER CONTENTS OF THREE FOODS AT VARIOUS a_w's AT 25°C

a_w	Water (% dry wt.)		
	Dried Mutton*	Dried Milk	Dried Soup
0·97	72·6	127	305
0·96	56·8	95·5	225
0·94	39·2	54·0	156
0·92	30·6	30·5	115
0·90	26·4	21·9	89·5
0·88	23·3	18·4	74·0
0·86	21·1	15·7	62·5
0·84	19·1	14·2	55·0
0·82	17·2	13·0	49·0
0·80	15·8	12·2	45·0

*Dry matter contains 32·4 per cent fat.

Although Table 7 records the plate counts after 5 days it may be noted that counts at 48 hr were not significantly less at a_w's of 0.96 and above, and after 24 hr only slightly less. At 0.94 a_w colonies reached diameters of 0.5 mm in 24-48 hr, and at 0.92 a_w in 48-96 hr. Colonies developing on agar at 0.999 a_w were observed to have a fuller development of the characteristic golden pigment than those appearing at lower a_w's.

IV. Discussion

The results provide clear evidence that the range of water activities permitting growth of food-poisoning strains of S. *aureus* was virtually independent of the nature of the solutes predominant in the medium. These solutes included sugars, some non-toxic salts, and the miscellaneous substances in bacteriological media and foods. Knowledge of the water activity in a particular food should

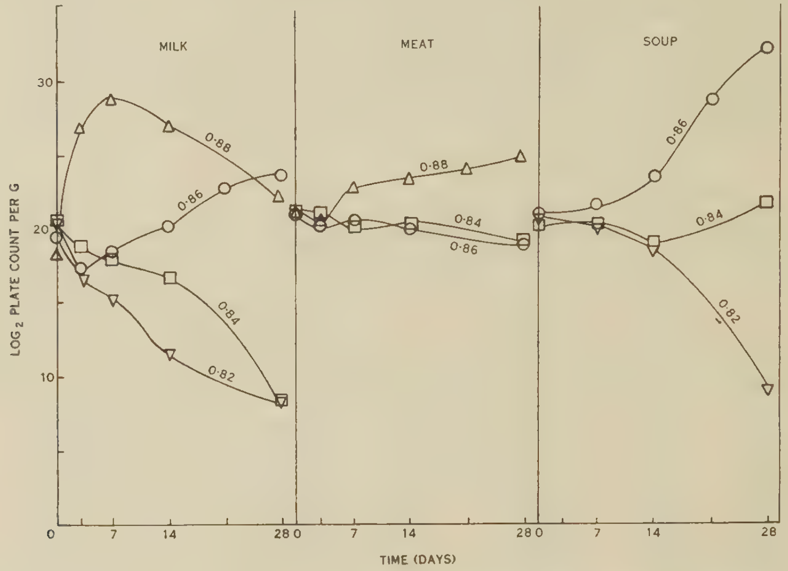

Fig. 4.—Growth curves for S. *aureus* inoculated into foods at different water activities. (Points are logarithmic means for separate experiments with strains F2B and 49/1974, both strains showing similar results.)

Table 7

EFFECT OF a_w OF THE PLATING MEDIUM ON THE PLATE COUNT OF *S. AUREUS*

Counts are means for triplicate plates inoculated with 10^{-6} ml of the broth culture, and incubated for 5 days at 30°C. Inoculum was strain 50/3002 grown for 18 hr at 30°C

a_w of Nutrient Agar used for Plate Count	Plate Count per ml × 10^{-6}	
	Nutrient Broth 0·999 a_w	C.Y.C. + Salts 0·960 a_w
0·999	435	111
0·990	426	117
0·980	426	114
0·960	414	116
0·940	421	81
0·920	405	38
0·900	0	0

therefore enable one to predict whether or not the substrate is sufficiently moist to support the growth of these bacteria.

The lower limit of a_w for growth at 30°C has been found to be about 0.86. This result was obtained at a temperature not far below the optimum for S. *aureus*, in the presence of an adequate supply of nutrients, and at approximately neutral pH levels. It is unlikely therefore that S. *aureus* will grow over a greater range of a_w under other environmental conditions. It is in fact more likely that factors such as unfavourable temperatures will restrict the range of a_w's over which growth occurs. Tomkins (1929), for instance, found that the range of a_w for germination of fungal spores was greatest at the optimum temperature.

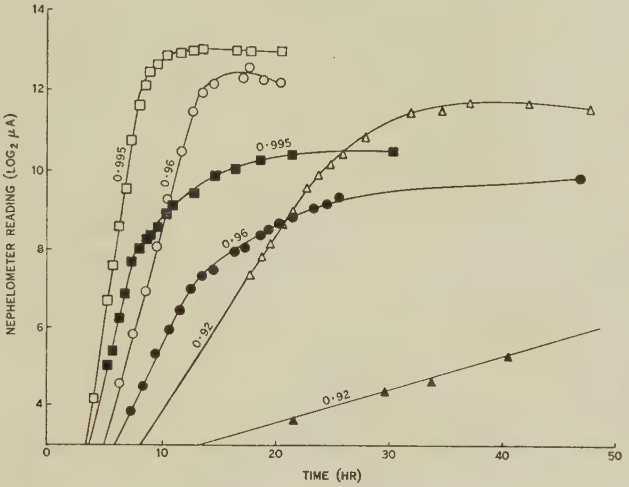

Fig. 5.—Growth curves of S. *aureus* strain 49/1974 under aerobic and anaerobic conditions at three water activities. Medium nutrient broth + 0.11m glucose + salts mixture to give desired a_w. Open symbols, aerobic; solid symbols, anaerobic.

An important feature of the experimental results was the uniformity between the strains. The 14 cultures were isolated from a wide range of sources and localities, but each has shown a very similar response under the various conditions of testing. The relative homogeneity of the group in water requirements indicates that other food-poisoning strains of S. *aureus* are likely to conform to the results obtained in the present experiments.

The expectation that other strains will behave similarly is strengthened by considering the results obtained by Segalove and Dack (1951). These authors studied the growth of another enterotoxin-producing strain (No. 161) on dried meat adjusted to water contents of 10, 20, 30, 40, 50, and 60 per cent. of the wet weight. They observed growth within 8 hr at 37°C at 40, 50, and 60 per cent. water and sometimes growth within 24 hr at 30 per cent. water. No growth was observed within 2 wk at 10 or 20 per cent. water contents. Although Segalove and Dack used meat samples varying appreciably in fat content a number of their samples had fat contents close to 30 per cent. If one assumes that 30 per cent. of the dry matter was fat and that the water vapour pressure isotherms are not dissimilar for the non-fat solids of beef, mutton, and pork, it is easy to deduce approximate values of a_w corresponding to the various water contents used by Segalove and Dack. Two such comparisons have been made, the first with the vapour pressure isotherm of dried mutton reported in this paper, and the second with an isotherm for beef muscle at $-1°C$ (Scott 1936). On the basis of these isotherms the a_w of the Segalove and Dack samples with 10 per cent. water was *c.* 0.70 or 0.71, and for the 20 per cent. water contents 0.885 or 0.872. Clearly S. *aureus* would not be expected to grow at 0.70-0.71 a_w, and the conditions would be marginal at 0.87-0.88 a_w. Segalove and Dack reported no growth within 2 wk. At 30 per cent. water the deduced a_w's are 0.945 or 0.935, at which levels the retarded growth observed by Segalove and Dack would be expected. At 40, 50, and 60 per cent. water contents the deduced a_w's are 0.967, 0.979, and 0.985 respectively, the values being virtually the same for both the isotherms. At these water contents Segalove and Dack observed growth within 8 hr, a result in complete harmony with those recorded in the present paper.

Hucker and Haynes (1937) failed to inhibit the growth of two food-poisoning strains by adding up to 50 per cent. of sucrose, and up to 12 per cent. of sodium chloride, to veal broth. This result is in accord with the present experiments, as the a_w's of these media would be about 0.93 and 0.92 respectively.

Nunheimer and Fabian (1940) studied nine food-poisoning strains of S. *aureus* in broth with various added solutes. After incubation for a week at room temperature they observed inhibition by 15-17.5 per cent. (w/v) NaCl, 50-60 per cent. (w/v) sucrose, and 35-45 per cent. (w/v) glucose. The corresponding a_w's are approximately 0.905 and 0.885 for the NaCl solutions, in close agreement with the present results. On the other hand the limiting concentrations with both sugars correspond to a_w's of the order of 0.95-0.93. Nunheimer and Fabian's results with the sugars are, therefore, not consistent with the limiting a_w's they found with NaCl. Their results also disagree with the present experiments, in which growth was consistently observed at a_w's down to 0.88 when sucrose was added to nutrient broth (Table 5).

The a_w of the selective medium of Chapman (1946) has been calculated and found to be close to 0.95, about 90 per cent. of the osmotic effect being due to the sodium chloride. It is clear from the present results that selective media with an even lower a_w could safely be devised for the enrichment and isolation of food-poisoning strains of S. *aureus*. At its present concentration, however, Chapman's medium has the advantage that it may be used under conditions where considerable evaporation from petri dishes occurs in incubators at 37°C. Even after the evaporation of one-third of its water content the medium would still have an average a_w of about 0.925, a value still acceptable for S. *aureus*.

In the present experiments the logarithmic growth phase has been well defined even at a_w's down to 0.90, and there has been no suggestion that more rapidly growing mutants have appeared in any of the cultures. The results obtained when plate counts were made at various a_w's (Table 7) also indicate that there is no need to invoke suggestions of training or selection of mutants as an explanation of growth at the lower levels of a_w. In some circumstances it appears that all cells grown at 0.999 a_w have the inherent ability to grow at a_w's at least as low as 0.92. At even lower levels of a_w there may simply be a reduced probability that a cell can reproduce successfully, such probability being affected by the endogenous resources of the cell as well as by its inherited capacity to reproduce in the environment concerned.

A feature of some interest is the occurrence of an optimum a_w close to 0.99. While the results do not explain the occurrence of this optimum it is clear that the effect is primarily osmotic, as both nutrient and non-nutrient solutes effect an increase in the growth rate when the a_w of the medium is decreased from 0.999 to 0.990. It is possible that S. *aureus* cells growing at 0.999 a_w have greater difficulty in maintaining a suitable intracellular osmotic pressure. In this connection it is pertinent to note that freezing points of unwashed cells centrifuged from nutrient broth of a_w 0.999 were of the order of -1.7°C. The cells therefore had an osmotic pressure some 20 times greater than the medium in which they were grown. The exact value cannot be stated as the deposited cells on which the freezing points were determined included an undetermined amount of the nutrient broth with a freezing point of -0.1°C. The correct determination of the osmotic pressure of the cells, the nature of the intracellular solutes in relation to a_w, and the amount of osmotic work performed by the cells in media of different a_w's are all matters which invite further study.

V. Acknowledgments

The author is grateful to Dr. R. H. Stokes for valuable advice on the methods for calculating the water activity of solutions containing several solutes. It is a pleasure also to acknowledge the assistance of Miss Betty J. Marshall who carried out most of the experimental work.

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WATER RELATIONS OF SALMONELLAE AT 30°C

By J. H. B. Christian[*] and W. J. Scott[*]

[*Manuscript received April* 30, 1953]

Summary

Sixteen strains of salmonellae have been grown in various media of known water activity (a_w) at 30°C. The reactions of 15 motile strains were very similar, whereas the single non-motile strain grew more slowly and over a smaller range of a_w's.

For the motile strains aerobic growth occurred in liquid media at a_w's between 0.999 and 0.945. In foods the lower limit for growth was slightly less. Anaerobic rates of growth were only slightly less than the aerobic rate at all a_w's. A large percentage of the cells could form colonies on agar media with a_w's as low as 0.96.

I. Introduction

Although outbreaks of food poisoning caused by salmonellae are known to be the result of infections rather than intoxications (Dack 1943), there is no precise information on the numbers of organisms which need to be ingested in order to produce the characteristic clinical symptoms. It is known, however, that the disease is likely to be severe and the incubation period short when the number of organisms ingested is large, and in many outbreaks there is evidence that the salmonellae had multiplied in the food concerned. It is important therefore to know the extent to which the growth of salmonellae will be dependent on the water contents of different types of foods.

In the preceding paper (Scott 1953) the importance of the water activity (a_w) in relation to the growth of *Staphylococcus aureus* is discussed, and this paper records the results obtained when 16 strains of *Salmonella* were studied in media in which the a_w was controlled. Similar studies do not appear to have been made previously, although some authors have studied the inhibition of growth by solutes such as sodium chloride. Severens and Tanner (1945) reported that 3 per cent. NaCl was the highest concentration permitting growth of unadapted strains of *S. pullorum* and *S. schottmuelleri* (*S. paratyphi B*). After adaptation for about 10 wk, growth was observed in media with 8 per cent. NaCl. In experiments with dried meat adjusted to various water contents Segalove and Dack (1951) reported that a strain of *S. enteritidis* required greater amounts of water for growth than *Staphylococcus aureus* or an alpha-haemolytic-type *Streptococcus*.

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II. Methods

The methods and media employed were the same as those described by Scott (1953) with the following minor modifications. Growth was measured nephelometrically at a_w's of 0.96 and higher when the media were sufficiently transparent. For several strains grown in the casamino acids + yeast extract + casitone (C.Y.C.) medium each μA of the nephelometer reading was equivalent to approx. 5×10^5 cells/ml at a_w's between 0.999 and 0.96. At lower a_w's and in opaque media growth was followed by viable counts on nutrient agar incubated for 48 hr at 30°C.

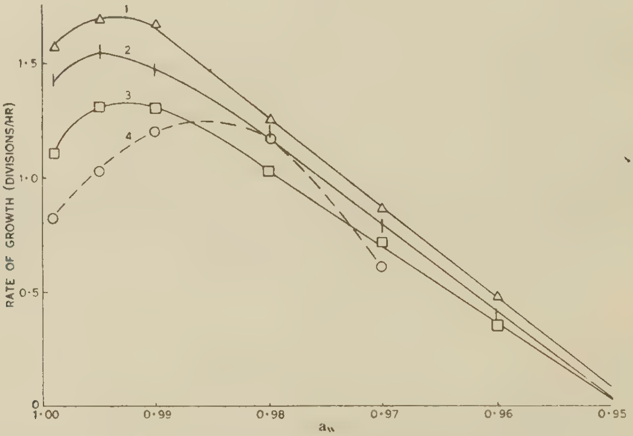

Fig. 1.—Relation between rate of growth and a_w for 16 strains of *Salmonella* in C.Y.C. medium with added salts.

Curve 1: Fastest growing motile strain S. *newport* (215).

Curve 2: Mean for 15 motile strains (vertical lines indicate mean ± its standard error).

Curve 3: Slowest growing motile strain S. *choleraesuis* (5735).

Curve 4: Aberrant non-motile strain S. *enteritidis* (3045).

Observations were made with the following 16 strains. Strains of S. *meleagris* (202), S. *typhimurium* (203), S. *oranienburg* (206), and S. *newington* (209) were isolated in this laboratory from Australian frozen eggs. The cultures were kindly identified by Miss N. Atkinson of the University of Adelaide. The authors are indebted to Miss Atkinson for also supplying a culture of S. *paratyphi B* (N.C.T.C. 3078) and the following five cultures which were recently isolated in Australia: S. *typhimurium* (210), S. *bovis-morbificans* (213), S. *anatum* (214), and S. *newport* (215). The following six N.C.T.C. cultures were kindly supplied by Dr. S. T. Cowan: S. *thompson* (5740), S. *choleraesuis* (5735), S. *dublin* (5766), S. *potsdam* (5744), S. *montevideo* (5747), and S. *enteritidis* (3045). All except the last-named culture were typical smooth motile salmonellae giving the characteristic biochemical reactions. S. *enteritidis* (N.C.T.C. 3045) was an aberrant non-motile strain which, in our hands, also failed to produce acid and gas from maltose. Although antigenically it was not distinguishable from S. *pullorum,* certain of its growth characteristics differed from those of strains of S. *pullorum* with which it was compared.

The cultures studied included the species which have most frequently been involved in outbreaks of food poisoning in the United Kingdom (Haines and Wilson 1947).

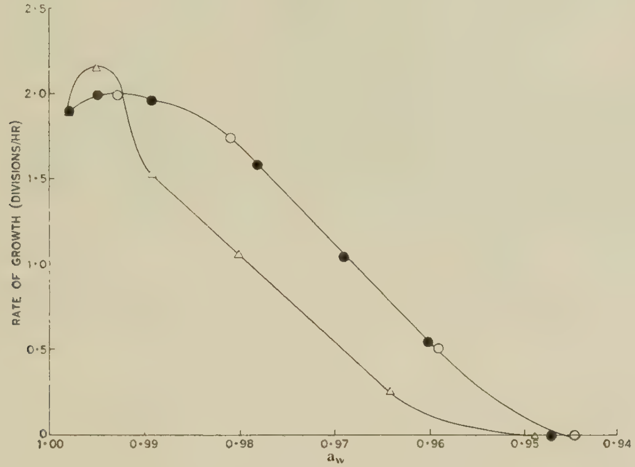

Fig. 2.—Relation between rate of growth and a_w for S. *oranienburg* (206) grown in B.H. medium using three methods for controlling a_w.

○, Water contents from vapour pressure isotherm for B.H. medium.

●, B.H. medium 9.25 g/1000 g H_2O and $NaCl:KCl:Na_2SO_4$ mixture in ratio of 5:3:2 moles.

△, B.H. medium 9.25 g/1000 g H_2O and sucrose.

III. Results

(*a*) *Growth in C.Y.C. Medium*

All strains were grown in C.Y.C. medium adjusted to various round values of a_w by a mixture of NaCl, KCl, and Na_2SO_4 in the molal ratio 5:3:2. The results are summarized in Figure 1. The 15 motile strains all showed a similar relation between rate of growth and a_w. Statistical analyses of the individual rates showed that these 15 strains were a homogeneous group with only a small variation between the fastest and slowest growing strains. For these 15 strains the mean rate of growth at 0.995 a_w was significantly greater than at 0.999 a_w. At a_w's below 0.99 the mean rate of growth decreased steadily with reduction of a_w, the rate at 0.96 being about 25 per cent. of the maximum rate. Linear extrapolation of the curves shows that growth is likely to be very slow at a_w's less than 0.95.

The broken curve in Figure 1 shows the results for the aberrant non-motile S. *enteritidis* N.C.T.C. 3045. For this strain the rates of growth were lower at all a_w's, the optimum was less well defined, and the rate of growth declined much more rapidly as the a_w was reduced below 0.98. Limited observations on three strains of S. *pullorum* suggested a similar type of response to that found for strain 3045.

(*b*) *Growth in Brain-heart* (*B.H.*) *Medium*

Growth of S. *oranienburg* was studied in B.H. medium using three different methods for controlling a_w. These were: firstly, by adjusting the water contents to the desired a_w according to the vapour pressure isotherm (Scott 1953, Table 1); secondly, by adding the appropriate amounts of the NaCl:KCl:Na_2SO_4 (5:3:2) mixture to a basal B.H. medium containing 9.25 g dry matter per 1000 g water; and thirdly, by adding sucrose to the basal medium of 9.25 g per 1000 g water. This basal medium contains one-quarter of the usual concentration of nutrients in B.H. medium and has an a_w close to 0.998.

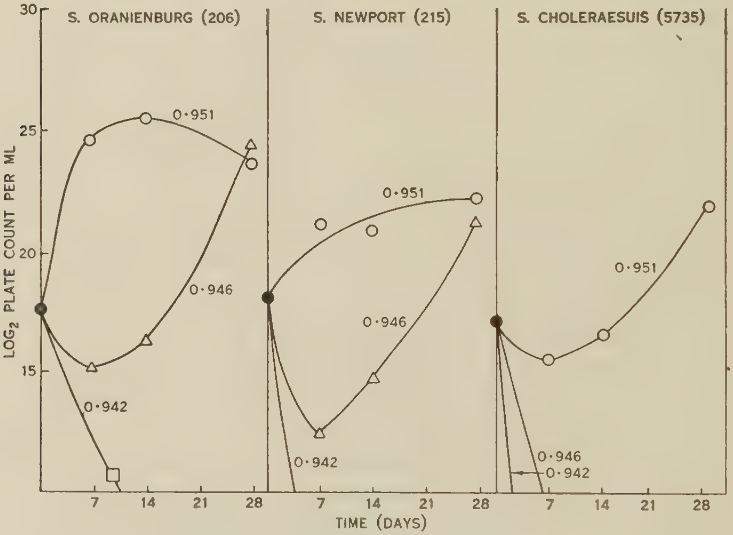

Fig. 3.—Limiting water activities for growth of three strains of *Salmonella* in B.H. medium.

The results (Fig. 2) show that the first two methods of controlling a_w gave a similar relation between the rate of growth and a_w. When sucrose was the principal solute there was some stimulation by the low concentrations of sugar at 0.995 a_w, but at a_w's less than 0.99 the rates of growth were lower than in the other two media. Growth was inhibited in all three media at a_w's between 0.95 and 0.94. The data show that the range of a_w's over which growth occurred was substantially the same when sugar, salts, or miscellaneous nutrients were the predominant solutes controlling the a_w.

(*c*) *Effect of* a_w *on the Lag and Yield of Cells*

As was shown for *Staph. aureus* (Scott 1953) reduction of the a_w below 0.99 led to an increase in the lag period and to a reduction in the total yield of cells. Estimates of the lag period were too variable for valid general conclusions to be drawn. In two media, however, the lag was less than 1 hr at a_w's above 0.99, slightly greater at 0.98, and *c*. 10 and 24 hr at a_w's of 0.97 and 0.96 respectively. Limited data on the yield of cells in C.Y.C. medium showed a decrease of about 50 per cent. as the a_w was decreased to 0.96. Evidence obtained in other media suggested that the maximum viable count decreased more rapidly as the a_w was reduced from 0.98 to 0.96.

(*d*) *Minimum* a_w*'s for Growth in Liquid Media*

Growth of three strains was followed in B.H. medium with water contents corresponding to three levels of a_w between 0.942 and 0.951. The results in Figure 3 show that all strains grew at a_w 0.951, that two strains grew also at a_w 0.946, and that none grew within 28 days at a_w 0.942. Under similar conditions S. *oranienburg* grew in nutrient broth at 0.945 a_w, but not at 0.940. Other experiments with this strain showed that the minimum a_w for growth was between 0.94 and 0.95 in C.Y.C. and B.H. media using either salts or sucrose to control a_w.

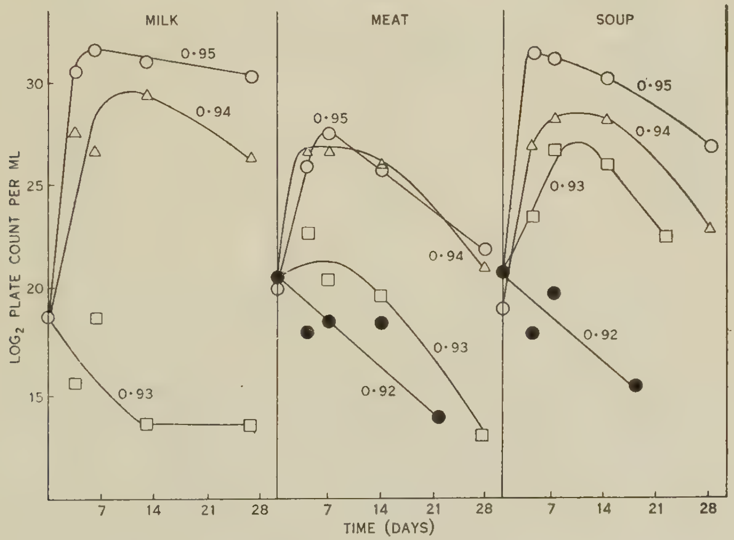

Fig. 4.—Limiting water activities for growth of S. *oranienberg* (206) in three foods.

(*e*) *Growth in Foods*

The growth of S. *oranienburg* was studied in dried meat, dried milk, and dried soup adjusted to water contents corresponding to the desired level of a_w. The results in Figure 4 show that growth occurred in all three foods at a_w's of 0.95 and 0.94. At an a_w of 0.93 the results differed according to the food. In milk the bacteria failed to grow, in meat there was perhaps a small increase, and definite growth occurred in the soup. There was no growth in either the meat or the soup at 0.92 a_w. Taken as a whole the results in foods are in fair agreement with those obtained in liquid media, although growth apparently occurred at somewhat lower a_w's in the foods. Reference to the data on the equilibrium water contents of the three foods (Scott 1953, Table 6) shows that at the limiting a_w's of 0.93-0.94 the water contents of the soup are some three to four times as great as those of milk and mutton. It is clear therefore that it is a_w rather than the water content which determines the availability of water for the growth of these bacteria.

(*f*) *Aerobic and Anaerobic Growth*

All the foregoing experiments were made under aerobic conditions and some comparisons were therefore made of aerobic and anaerobic growth at various a_w's. The average results for three strains are given in Figure 5. It is clear that the anaerobic rates of growth were only slightly less than the aerobic rates at all a_w's tested, and that the lower limits for growth were virtually the same under both conditions.

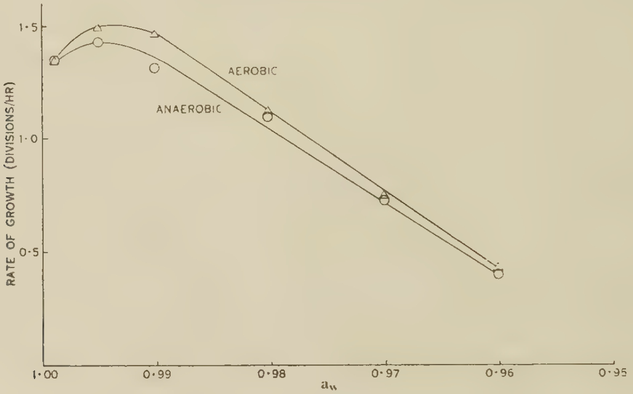

Fig. 5.—Comparison of aerobic and anaerobic rates of growth for three strains of *Salmonella* in C.Y.C. medium adjusted to various levels of a_w with added salts. The points are the means of at least two determinations for each of the three strains *S. newport* (215), *S. oranienburg* (206), and *S. choleraesuis* (5735).

(*g*) *Effect of* a_w *on the Plate Count*

Some experiments were made with one strain using nutrient agar adjusted to various a_w's with the NaCl-KCl-Na$_2$SO$_4$ mixture mentioned previously. The results are summarized in Table 1. For cells grown in nutrient broth of 0.999 a_w χ^2 tests showed that the number of cells forming colonies at 0.96 a_w was significantly less than at the higher a_w's. For cells grown at 0.96 a_w the counts were consistent with homogeneity at all a_w's. It may be concluded therefore that under some conditions virtually all the cells in a culture have the capacity to grow at a_w's as low at 0.96. Even for cells grown in nutrient broth of 0.999 a_w there were still some 40 per cent. of the cells which formed colonies at 0.96 a_w.

IV. Discussion

The results with salmonellae confirm the finding previously reported for *Staph. aureus* (Scott 1953) that the range of a_w's permitting growth is substantially independent of the type of solute predominant in the medium. For the salmonellae, however, the range of a_w's in which growth occurs is much less than for the staphylococci. In liquid media the lowest a_w at which growth was observed was close to 0.945, whereas in foods growth occurred at slightly lower a_w's. In liquid media there can be little uncertainty about the uniformity of a_w throughout the medium, but in the moist foods there is greater difficulty in ensuring and maintaining uniform distribution of the water added. It is suggested therefore that the results obtained in the liquid media may be the more reliable. It is, however, not possible to reject the idea that growth on solid media may occur at slightly lower a_w's than in liquids.

Table 1

EFFECT OF a_w OF THE PLATING MEDIUM ON THE PLATE COUNT OF *S. ORANIENBURG*

Counts are means for four plates incubated for 5 days at 37°C

a_w of Nutrient Agar Used for Plate Count	Plate Count	
	Nutrient Broth 0·999 a_w *	C.Y.C. + Salts 0·960 a_w †
0·999	258	56
0·995	246	69
0·990	244	75
0·980	207	70
0·970	248	73
0·960	104	63

* Plates inoculated with 2×10^{-8} ml of culture grown for 16 hr at 30°C.
† Plates inoculated with 2×10^{-6} ml of culture grown for 4 days at 30°C.

The uniformity with which the various strains have reacted is an important feature of the results and it seems reasonably safe to assume that other motile salmonellae would have similar water requirements. This supposition is further strengthened by the fact that the organisms studied have included both recent and old isolates from a variety of sources. The different results obtained with the non-motile *S. enteritidis* 3045 are of interest, although there is no evidence that the reactions of this organism are typical of non-motile salmonellae generally. From a practical point of view the important feature is that the typical motile organisms exhibit the greater tolerance as the a_w is reduced below 0.97.

It is of some interest to compare the limiting a_w of *c.* 0.945 in the present experiments with the approximate a_w's which may be deduced from the limiting conditions reported by other workers. Severens and Tanner (1945) found no growth of unadapted salmonellae above 3 per cent. NaCl, but after adaptation growth occurred in up to 8 per cent. NaCl. The latter solution has an

a_w close to 0.95. Segalove and Dack (1951) inoculated *S. enteritidis* into dried meat with various water contents for which the approximate corresponding a_w's have been deduced by Scott (1953). At 50 per cent. water contents scant growth occurred on one sample within 24 hr, but no growth within the same time at 40 per cent. water contents. The corresponding a_w's were *c.* 0.979 and 0.967 for meat samples without added salt. Some of the samples with added salt may, however, have had a_w's as much as 0.02 less and this is doubtless the explanation for Segalove and Dack's finding that the results depended on the salt content. In general the results of Segalove and Dack are consistent with those in this paper, although it might be expected that they would have observed growth in some samples with 40 per cent. moisture if observations had been continued beyond 24 hr.

There are some grounds for the belief that the water requirements of most coliform bacteria may be similar to those of the salmonellae. With *E. coli*, for example, there is general agreement (Foda and Vaughn 1950) that 5-8 per cent. NaCl is needed to inhibit growth. These concentrations correspond to a_w's of about 0.97 and 0.95 respectively. Foda and Vaughn also point out that most strains of *Aerobacter aerogenes* do not tolerate more than 6.5 per cent. (w/v) NaCl (a_w 0.96), and unpublished observations by one of us (J.H.B.C.) have shown that the Hinshelwood strain of *A. aerogenes* is able to grow at 30°C at a_w's down to about 0.94.

The results reported in this paper have been obtained with unadapted cultures and no attempt has been made to determine whether salmonellae could be adapted or selected to grow at even lower a_w's. Although Severens and Tanner (1945) reported that salmonellae acquired permanently increased tolerance to NaCl as a result of selection, the maximum concentration of salt tolerated after adaptation was 8 per cent. (a_w 0.95). Similarly the results of Doudoroff (1940) and of Foda and Vaughn (1950) show that most coliform organisms could not be trained to grow in NaCl concentrations with a_w's less than about 0.945. The only exception was a group of *Aerobacter* strains which Foda and Vaughn isolated by enrichment from olive brines, and which they found could grow in the presence of up to 14.5 per cent. (w/v) NaCl, the corresponding a_w being close to 0.91. The possibility of training or selecting salmonellae to grow at a_w's below 0.94 certainly exists, but there are no indications that any such attempts are likely to be successful. On the other hand the present experiments have always shown a well-defined logarithmic phase at 0.96 a_w and there has never been any suggestion that more rapidly growing variants had appeared in any of the cultures. A decisive answer regarding the chances of adapting salmonellae to low a_w's must await further experiments.

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A POLYHEDRAL VIRUS DISEASE OF A PASTURE CATERPILLAR, *PTEROLOCERA AMPLICORNIS* WALKER (ANTHELIDAE)

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Summary

A new virus of the genus *Borrelina,* causing a polyhedral disease in larvae of the anthelid moth *Pterolocera amplicornis,* is described. The polyhedral bodies are small and unusually resistant to alkaline solution. Electron micrographs reveal the presence of rods, spheres, and rods projecting from spheres in alkali-digested polyhedra. The relations between these bodies are not clear.

I. Introduction

Recent reviews (Steinhaus 1949*a*, 1949*b*; Smith and Wyckoff 1951) have shown that viruses of the genus *Borrelina* Paillot, causing polyhedroses in at least 17 families of larval Lepidoptera, are widespread in the northern hemisphere. Examination of a number of larval Lepidoptera from laboratory cultures and field collections in Australia during the last 3 years has failed until now to reveal a polyhedral disease. A polyhedrosis in field-collected larvae of the anthelid *Pterolocera amplicornis* Walker has now been discovered in the vicinity of Canberra. This paper presents some details of the host, the disease, and the pathogen.

II. The Insect Host

The family Anthelidae is composed of medium to large moths, and is confined to the Australian region, including New Guinea and the Celebes. The genus *Pterolocera* Walker contains only two described species. The type species, *P. amplicornis,* which has a wingless female, occurs from southern Queensland to Tasmania and in South and south-western Australia, and is a pest of native grass pastures. The species as at present constituted is extremely variable. *P. isogama* Turner has a winged female and occurs in south-western Australia. Its larvae feed on *Acacia acuminata* Benth. The desirability of including both species in the same genus is open to doubt.

Although of some economic importance (see Evans 1943), nothing of the life history of *P. amplicornis* has been published. At Canberra it completes a single generation each year. Adults, which do not generally live more than 24 hr, have been take in mercury vapour light traps from the end of February until early April, with the peak in the latter half of March (Fig. 1). Eggs laid in autumn hatch in about 7 wk at laboratory temperatures, and the larvae develop slowly during the winter. Larval growth is accelerated in early spring and mature larvae (Plate 1, Fig. 1) occur during October and November. They are hairy caterpillars, just over 2 in. long, and pupate in the soil in shallow tunnels in which they spin a silken cocoon. The prepupal and pupal periods occupy about 16 wk.

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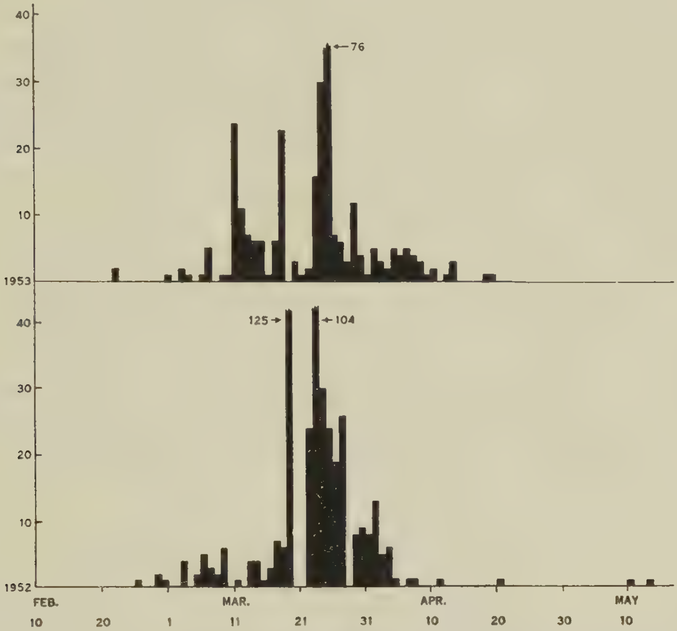

Fig. 1.—*Pterolocera amplicornis* moths collected in mercury vapour light trap, Canberra, 1952-53.

III. The Disease

Larvae infected with polyhedrosis were first noticed early in October and occasional infected specimens were found until the majority of larvae had pupated. Less than 1 per cent. of larvae collected in the field were infected.

When larvae were fed on grasses sprayed with a suspension of polyhedra, symptoms appeared in some, but not all, larvae in about 4 days at laboratory temperatures. The first symptom is a loss, starting from the posterior end of the body, of the typical silvery appearance, owing to the deposition of a dark pigment in the cortex of the long unpigmented hairs on the dorsal surface. This darkening is continued anteriorly until the entire body is darker than normal (Plate 1, Fig. 1). A similar loss of the silvery appearance of the hairs occurs when these are immersed in alcohol. At the same time the infected insects become sluggish, flaccid, and do not curl up when handled as do the uninfected specimens. The cuticle of the moribund larva is fragile and when punctured permits the escape of a thick, creamy fluid containing very large numbers of polyhedra and bacteria. Insects in this condition were found in the field on the low grass upon which they feed or on the soil surface.

The histopathology is similar to many polyhedroses previously described. The polyhedra are observed first in the nuclei of fat body, hypodermis, tracheal epithelium, and haemocytes. Eventually, all tissues are attacked, with the exception of gut epithelia, malpighian tubules, silk glands, nervous tissue, and muscular tissue. As the number of polyhedra increases in the nucleus, the residual chromatin is clumped in the centre, and the nucleus increases greatly in size (Plate 1, Figs. 2 and 3) until it eventually ruptures, liberating the polyhedra into the haemocoele. Smears of polyhedra from *Pterolocera* are uncoloured by Feulgen's stain and by methyl green. After treatment with alkali the polyhedra stain weakly green with the Unna-Pappenheim stain. After treatment with Schweitzer's reagent they stain with either methyl green or pyronin when these are used separately.

It has not been possible to test the infectivity of the disease to a variety of other Lepidoptera. However, mature larvae of a noctuid (*Persectania ewingii* (Westw.)) were in culture at the same time as infected larvae of *P. amplicornis* occurred in the field. Attempts to infect *P. ewingii* by feeding them grass sprayed with the polyhedra were unsuccessful. In view of the observation of Smith and Xeros (1952), that cross infection may occur more readily between viruses from insects that are not closely related than between those from closely related hosts, an attempt was made to infect the larvae of *Cacoecia australana* (Lewin) (Tortricidae) by feeding foliage sprayed with a heavy suspension of polyhedra. No infections resulted.

IV. The Polyhedral Body and the Virus

Infection of larvae is readily diagnosed under the dark-field microscope (Plate 1, Fig. 5). The polyhedral bodies are easily distinguished (Plate 1, Fig. 4) from the less highly refractive bacteria which are present in large numbers during the later stages of the disease. The polyhedral bodies are smaller than any hitherto described, so that accurate measurements of their dimensions and determination of their shape is possible only by means of electron micrographs (Plate 2, Figs. 6, 7, and 8). The polyhedra are irregular in shape and their diameters range from 0.7 to 1.3 μ with a mean of about 1.0 μ. They scatter electrons so effectively that no internal details can be observed with a 50 kV electron microscope.

Early attempts to free the virus from the polyhedra proved the latter to be capable of resisting attack by 0.01M NaOH solution (pH 12) for 2 hr at room temperature (Plate 2, Figs. 7 and 8). Plate 3, Figure 9, shows polyhedra treated for 60 min at 56°C with 4 per cent. Na_2CO_3 ($\cong$ 0.38M, pH $\cong$ 11.0) while standing on a collodion supporting membrane. It is apparent that the dissolution of the nucleoprotein component is only partially complete. Plate 3, Figure 11, and Plate 4 show polyhedra from which identical treatment removed almost all the nucleoprotein. The polyhedra of Plate 3, Figures 10 and 12, were treated by suspension in 4 per cent. Na_2CO_3 for 180 min at 50°C. The length of the shadow cast by a completely empty membrane (Plate 4, Fig. 14) suggests that the membrane is only 30 Å thick. This must be regarded as a lower limit as it is possible that the membrane is partially buried in a layer of breakdown products deposited on the collodion supporting film. Some membranes exhibit ridges (Plate 4, Fig. 16), not previously recorded, that probably indicate the edges of the crystals.

The dissolution of the nucleoprotein of the polyhedra permits the escape of both thin rods (3500 Å long by 1500 Å wide) and thick rods (3200 Å long by 1700 Å wide) and of spheres (1400 Å in diameter) (Plate 3, Figs. 10-12; Plate 4, Figs. 13-16). Both the rods and the spheres appear to be flattened, which may be due to collapse on drying and to their being partially embedded in a contaminating layer of breakdown products deposited on the supporting membrane. Little light is shed by the micrographs on the relationships between these three types of bodies. Examples are found of thin rods projecting from spheres (Plate 4, Fig. 14) but none of the micrographs suggests that the thick rods consist of bundles of thin rods. The number of rods and spheres contained in each polyhedron is also obscure. Plate 3, Figure 10, indicates that some six or more thick rods appear in each polyhedron, and Plate 4, Figures 13 and 15, suggests that the polyhedra each contain numbers of thin rods. These two micrographs, however, are atypical and most fields contained few thin rods. A few thin rods and flattened spheres are visible in untreated specimens (Plate 2, Fig. 6) and also in preparations subjected to 0.01M NaOH for 120 min at room temperature, which is apparently insufficient to dissolve the polyhedra (Plate 2, Figs. 7 and 8).

On the basis of the above description it is evident that the virus has not been previously described and the name *Borrelina anthelus* sp. nov. is therefore proposed for it.

V. Discussion

The virus causing the polyhedrosis described in this paper is the first of the genus to be recognized from Australia. It is also the first recorded in the family Anthelidae, although many polyhedroses of other noctuids have been reported.

The relationships of species of the genus *Borrelina* have not been thoroughly examined and the criteria for interspecific comparisons are few. *B. anthelus* sp. nov. has small polyhedra like those from the hymenopteran *Gilpinia* (Bird 1952) and the virus elementary bodies occur in both spherical and rod-shaped forms. However, the thick rods are not obviously composed of the thin rods as they are in all previously described polyhedral diseases. Hughes (1950) considers that there is a correlation between the size of the polyhedra and the number of virus bundles they contain. The *Pterolocera* polyhedra appear to confirm this generalization.

Smith and Wyckoff (1951) mention that polyhedra from different species differ greatly in their resistance to alkaline treatment, but the most resistant that they studied were dissolved by 5 min treatment with 4 per cent. Na_2CO_3. The polyhedra of *Pterolocera* withstand this concentration for 30 min and a treatment of at least 60 min at 56°C was necessary to dissolve them completely. *B. anthelus* is thus far more resistant to alkali than any previously described species.

Rods and spheres have been found by Smith and Wyckoff (1951) in polyhedra causing different diseases. Bergold (1950), however, found both to occur in a single disease and considered they represented stages in the life cycle of the viral organism. A similar conclusion was reached by Bird (1952) who studied the polyhedral disease of the hymenopteran *Gilpinia*; in this virus the spheres were found in early stages of infection and rods in later stages. In the *Pterolocera* polyhedra the spheres and rods have been found together in almost every preparation. The conclusion of Hughes (1952) in respect to the granulosis disease of the looper caterpillar *Sabulodes caberata,* that the relationship between the spheres and rods has not yet been clarified, is further exemplified by the observations on the virus of *Pterolocera.*

It is not known whether the polyhedral disease of *Pterolocera* caused greater destruction in 1952 than in previous years. The season was wetter than usual, and 5.65 in. of rain were recorded in October, whereas the monthly average over 27 years is 2.40 in. (maximum 6.59 in. in 1934, minimum 0.34 in. in 1940). The percentage of infected larvae in the field was somewhat higher in parts of the pastures near surface water. From general observations the incidence of the disease appeared to be higher than in previous years. It is probable also that the larval population was higher in 1952 than in the previous year. Light trap records show that populations of *Pterolocera* moths in March 1952 were very much higher than in the previous year. The resultant larval population may have therefore been larger and this could have contributed to a higher incidence of the disease. It is evident, however, that the epizootic did not reduce the population in 1953 to a level below that of the previous season (Fig. 1).

VI. ACKNOWLEDGMENTS

Thanks are due to Miss P. Sinclair, Division of Entomology, C.S.I.R.O., who brought the first infected larvae to our attention, to Mrs. M. J. Bennetts for the histopathological preparations, and to Mr. D. Wilson for the photomicrography. Meteorological data were kindly furnished by the Australian Forestry School, Canberra.

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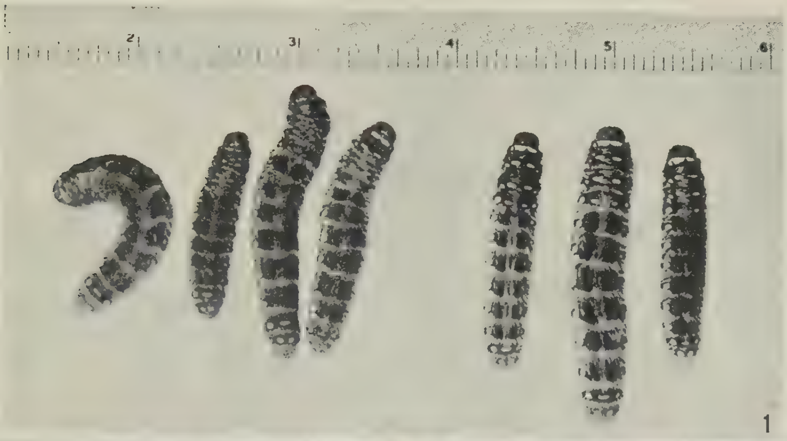
POLYHEDRAL VIRUS DISEASE OF A CATERPILLAR



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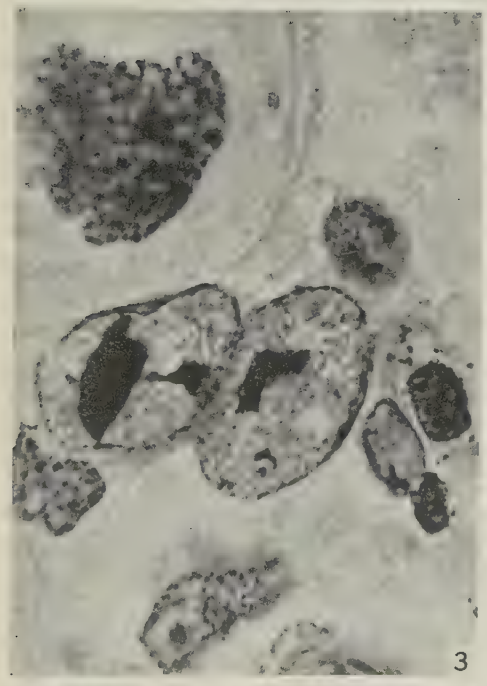

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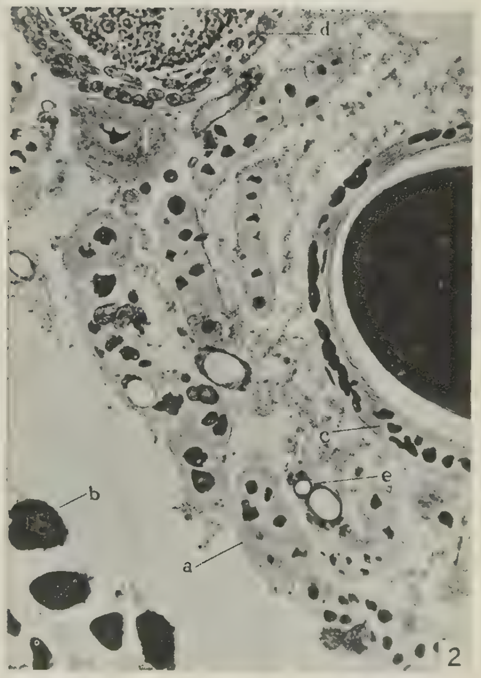

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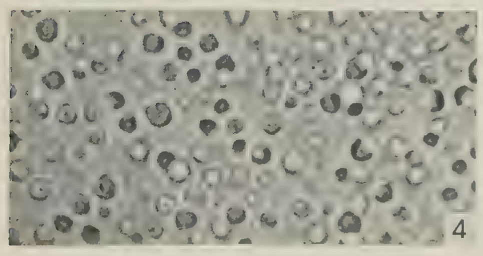

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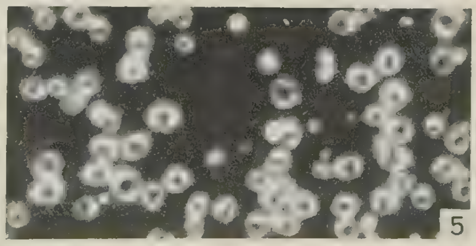

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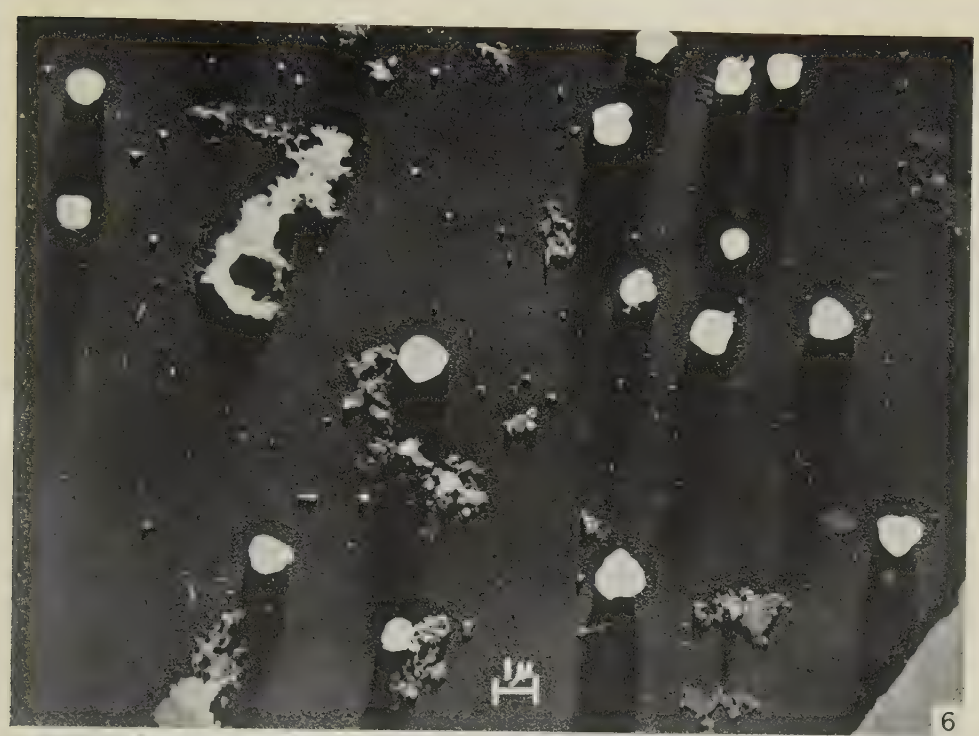

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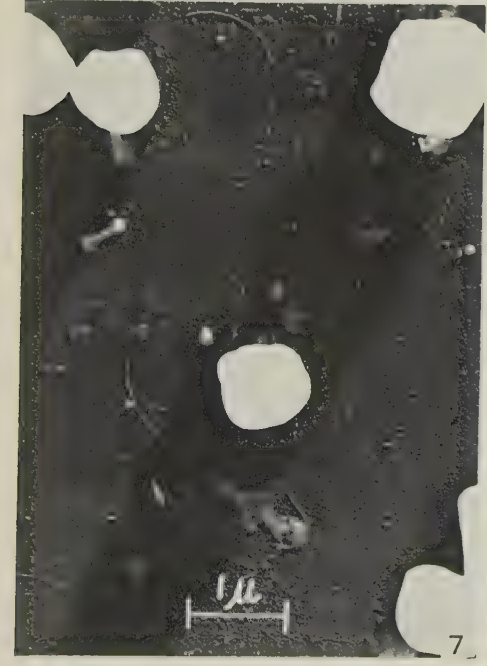

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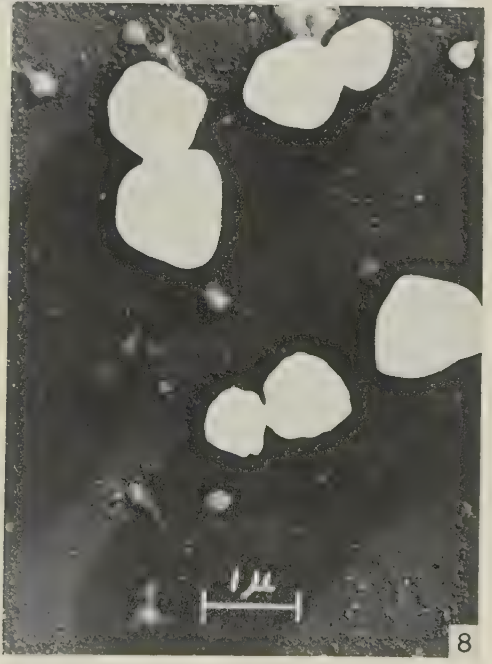

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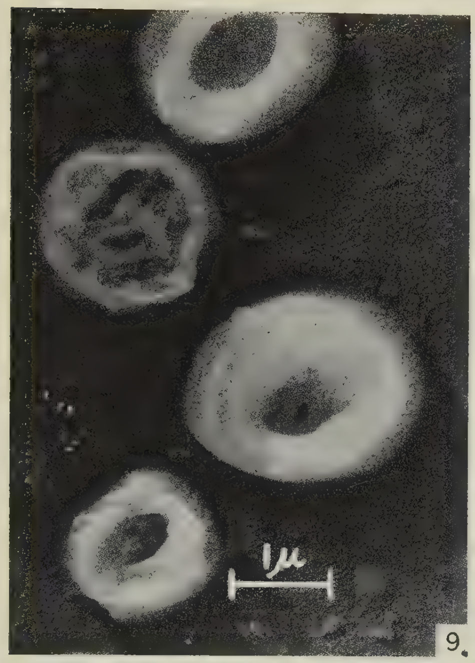

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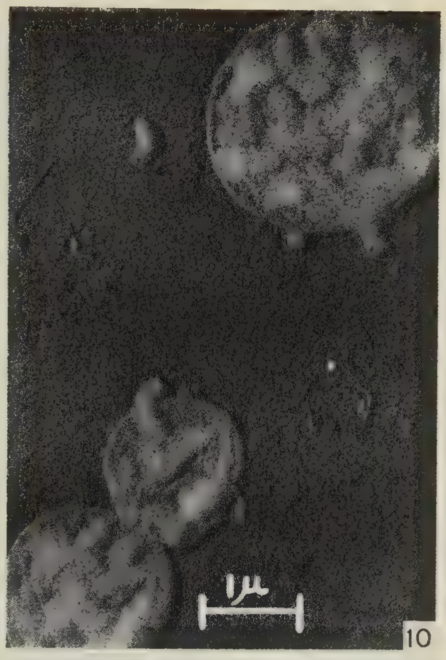

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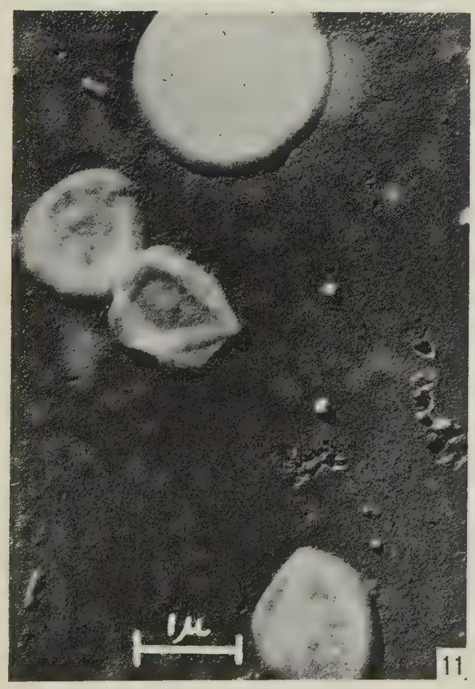

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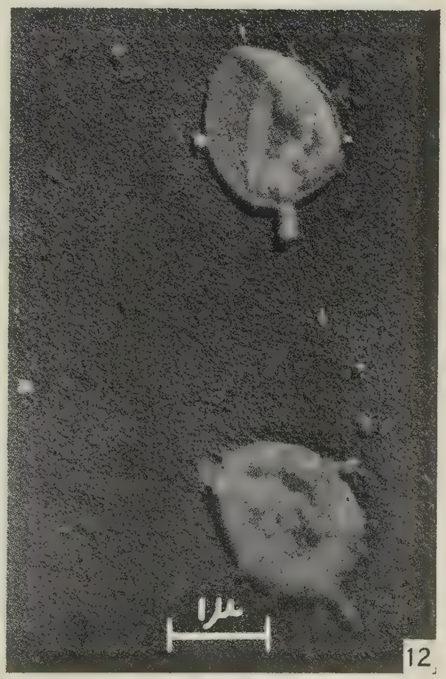

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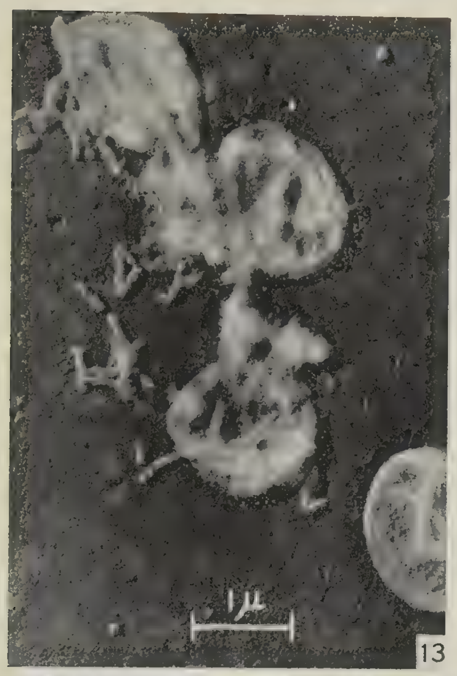

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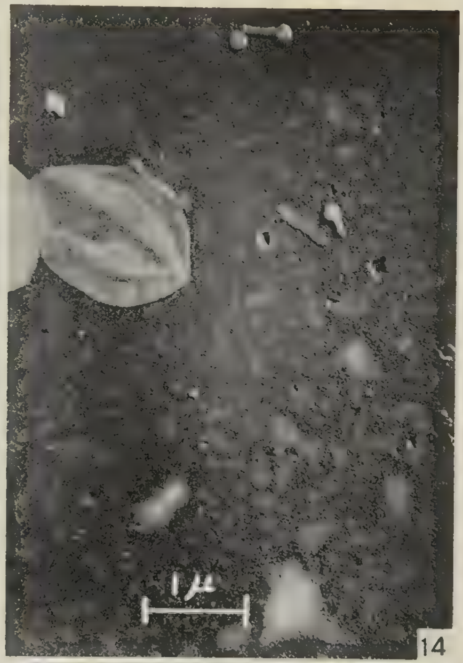

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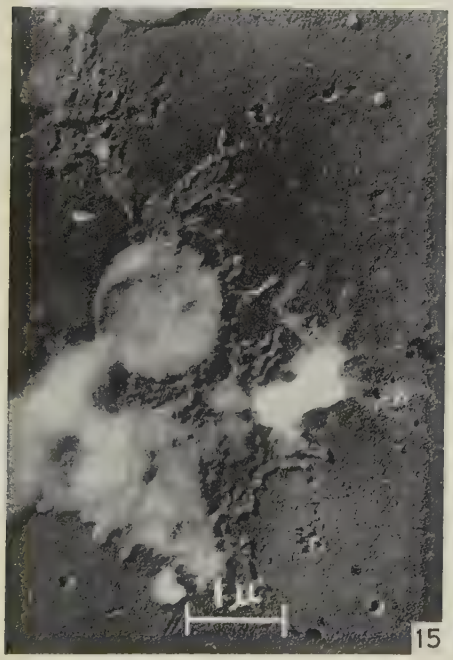

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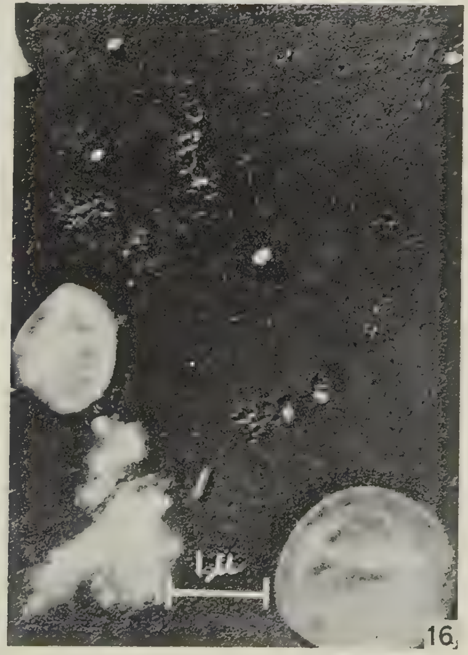

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Explanation of Plates 1-4

Plate 1

Fig. 1.—Mature larvae of *Pterolocera amplicornis.* Three normal larvae (right) and three larvae infected with polyhedrosis. Note dullness of body hairs of the infected larvae.

Fig. 2.—Histopathology of *P. amplicornis* infected by polyhedral disease. Note hypertrophied nuclei in fat body and in tissue surrounding the testes. (*a*) Fat body, (*b*) muscle, (*c*) silk gland, (*d*) testes, (*e*) trachea.

Fig. 3.—Same as Figure 2 under oil immersion objective. Polyhedra can be distinguished in the hypertrophied nuclei.

Fig. 4.—Polyhedra in haemolymph of infected larva. Mean diameter of polyhedra approximates 1.0 μ.

Fig. 5.—Same as Figure 4 with dark field condenser.

Plate 2

Fig. 6.—Untreated polyhedra from infected larvae of *Pterolocera amplicornis.* Note presence of thin rods and flattened spheres. Shadowed with uranium. Ratio of shadow length to particle height is 6.

Figs. 7 and 8.—Polyhedra apparently unaffected by treatment with 0.01M NaOH solution for 120 min at room temperature. Thin rods and flattened spheres present. Shadow ratio is 4.

Plates 3 and 4

Fig. 9.—Incompletely digested polyhedra after 60 min treatment with 4 per cent. Na_2CO_3 solution at 56°C while standing on the collodion supporting film. Shadow ratio is 4.

Figs. 10 and 12.—Polyhedra after suspension in 4 per cent. Na_2CO_3 solution for 180 min at 50°C. Shadow ratio is 4.

Figs. 11, 13-16.—Polyhedra after 60 min treatment with 4 per cent. Na_2CO_3 solution at 53°C. Polyhedra treated while standing on the collodion supporting film. Note thin rods in Figures 11 and 14 and flattened spheres in Figures 11, 13, 14, 15, and 16. Shadow ratio is 4.

HEALING OF GUT WOUNDS IN THE MOSQUITO *AEDES AEGYPTI* (L.) AND THE LEAFHOPPER *OROSIUS ARGENTATUS* (EV.)

By M. F. Day* and Margaret J. Bennetts*

[*Manuscript received May* 1, 1953]

Summary

Healing of wounds made by puncturing the replete midgut of adult female *Aedes aegypti* occurs without the intervention of the haemocytes, which play an important role in all previously described examples of wound healing in insects. An eschar is formed on the cuticle within 24 hr, apparently as the result of haemolymph coagulation, but initial closure of the wound appears to be the result of the activity of the midgut musculature and healing to be due to the regenerative powers of the midgut epithelium. Survival as high as 80 per cent. is attained when the gut of engorged females is punctured. The midgut wound may remain gaping for 24-48 hr, and in one case was unhealed at 54 hr. Ingested mammalian erythrocytes generally do not escape into the mosquito haemocoele, because they are agglutinated in the midgut and are held together by a peritrophic membrane. In adult *Orosius* the midgut epithelial cells are polyploid and regenerate slowly. The early stages of the healing process are therefore performed by haemocytes. These are present in small numbers only, and undergo active mitosis to form the wound tissue. The significance of these observations on some results on the transmission of viruses by mosquitoes and leafhoppers is discussed.

I. INTRODUCTION

There is substantial evidence that plant viruses transmitted by leafhoppers are ingested with the infected host sap, penetrate the midgut, reach the salivary glands through the haemocoele, and are reinjected with the insect's saliva into a second host plant (Storey 1939). Certain strains of *Cicadulina mbila* (Naude), the leafhopper vector of maize streak virus, were incapable of transmitting the virus, but Storey (1933) was able to convert insects of an "inactive" strain into vectors by puncturing the midgut. He therefore suggested that the midgut constituted a barrier to the penetration of the virus. Following this demonstration, the concept of the midgut as a barrier to virus infection was considered also in mosquitoes. Merrill and Ten Broeck (1935) were able to increase the ability of *Aedes aegypti* to transmit eastern equine encephalomyelitis virus by puncturing the midgut of this mosquito. Philip (1948) also studied the effect of gut puncture on the transmission of *Rickettsia burneti* by *Aedes aegypti.*

Despite these studies of virus-vector relationships by operations on the mosquito and leafhopper midgut, no work has been done on the rate of healing of the wounds or, in fact, on any aspect of the healing process in these insects. In the cockroach *Periplaneta,* healing of gut wounds is efficiently performed by the combined action of haemocytes and regeneration of the gut epithelium (Day

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1952). It might be expected that the process described in *Periplaneta* would have general application to other insects. However, preliminary observations on the mosquito and the leafhopper indicated that this was not so. A more detailed study of wound healing in these insects was therefore necessary for better understanding of the use of gut puncturing in the study of virus-vector relationships of *Aedes* and *Orosius*.

II. MATERIALS AND METHODS

(*a*) Aedes aegypti (*L.*)

A colony of *Aedes aegypti* was maintained at about 27°C and 60-80 per cent. R.H. Adults were fed on a guinea pig, and the midgut was punctured 1-4 hr after feeding.

Using a finely sharpened steel needle, the wounds were made by puncturing the abdominal pleura of engorged females. Preliminary observations confirmed the statement of Merrill and Ten Broeck (1935) that survival of operated females was considerably lower if the midgut was punctured when it contained no blood. Survival in later series approximated 80 per cent., and some adults lived for 3 wk before being fixed for histological examination in the alcoholic modification of Bouin's fluid. No attempt was made to keep the mosquitoes alive longer than 3 wk. Serial sections (10 μ) of 165 specimens were studied following fixation at various times from 1 hr to 14 days after wounding.

(*b*) Orosius argentatus (*Evans*)

Colonies of this leafhopper have been maintained in the glass-house for work on virus transmission (see Helson 1942 for methods). Operations similar to those described by Storey (1933) were performed on 91 adults, using carbon dioxide anaesthesia. Survival approximated 40 per cent., and serial sections were made of 32 specimens fixed from 1 to 12 days after the operation.

III. OBSERVATIONS ON AEDES AEGYPTI

Healing of midgut wounds occurs in *A. aegypti* by an efficient but apparently simple mechanism. No tissue other than the simple epithelium and the gut musculature is involved. The first stage of healing is the result of a contraction of the gut musculature in the vicinity of the wound. This normally has the effect of stopping the passage of solid contents of the gut into the haemocoele, and has been observed up to 29 hr, but not at 45 hr, after wounding. In only one case were ingested erythrocytes observed to have passed into the haemocoele. This was in a mosquito operated upon at least an hour after the blood meal, and fixed 2 hr after puncturing. The muscular spasm produces a marked change in the shape of the epithelial cells surrounding the wound (Plate 1, Fig. 1). After feeding, the epithelial cells are flattened, but gradually regain their columnar shape as digestion of the erythrocyte mass proceeds. The muscular spasm in the immediate vicinity of the wound rapidly causes the epithelium to assume a columnar shape and this is retained until the muscles relax.

It seems likely that the ability of the engorged midgut to contract provides a partial explanation of the higher survival rates observed in engorged females than in unfed females. In addition it is true that fewer cells are damaged in puncturing the gut of an engorged female than in performing the same operation on the gut of an unfed mosquito.

Before relaxation of the gut musculature occurs, proliferation of the midgut epithelium begins, and continues for several days until epithelial continuity is established (Plate 1, Fig. 2). Excess proliferation may result in reduction in size of the nearby cells. Recovery from this apparent exhaustion may be incomplete 14 days after wounding. There is little necrosis of the epithelial cells, even those only one or two cells removed from the wound. However, vacuolization may occur in some epithelial cells (see Plate 1, Fig. 3).

No sign of epithelial damage could be detected in complete serial sections of three out of 11 insects fixed 3 days after puncturing, and in eight insects out of 12 fixed 7 days after puncturing. Healing must therefore occur with remarkable completeness, doubtless correlated with the normal regenerative ability of the midgut epithelium.

The healing process just described may be complicated by several factors. For example, midgut contents (partially digested erythrocytes) may protrude into the wound and may delay epithelial regeneration (Plate 1, Fig. 3). In one specimen normal epithelial regeneration was modified by the formation of a plug of relatively unorganized cells that persisted for 7 days (Plate 1, Fig. 4). Healing may be delayed for reasons not understood. For example, Plate 1, Figure 5, shows a gaping wound found 54 hr after the wound had been made. In this specimen the cuticle wound had healed normally, and in two other mosquitoes punctured and fixed at the same time the midgut wound was normally and completely healed.

In only one of the 165 specimens examined were haemocytes involved in the healing process. In this example, groups of haemocytes extended from the cuticle wound to the gut wound, presumably along the path taken by the puncturing needle. They showed no tendency to accumulate at sites of necrosis, as they do in *Periplaneta* (Day 1952).

Methods are not available for measuring the strength of the regenerated tissue in the *Aedes* midgut. Effective repair may be considered complete when the gut is able to withstand the ingestion of a second blood meal. Mosquitoes that were disturbed before ingestion was complete were wounded and then permitted to feed a second time. Two of these took a second blood meal 5 hr after wounding, two more 24 hr after wounding, and one fed 3 days after wounding. When the second blood meal was taken 5 hr after wounding, the erythrocytes escaped into the haemocoele, but this was not observed when the second meal was taken 24 hr after wounding.

Healing of the cuticle wounds likewise proceeded efficiently without the intervention of haemocytes. An eschar was frequently formed of necrotic tissue and presumably also consisted of coagulated haemolymph. Plate 1, Figure 6, illustrates a fairly extensive cuticle wound involving also some necrotic fat-body. Occasionally adhesions between the cuticle and midgut were found, but did not appear to hinder the process of repair.

IV. Observations on Orosius argentatus

In only 25 per cent. of *O. argentatus* operations was the gut punctured. If *C. mbila* anatomy is similar to that of *O. argentatus*, which is probable, it seems likely that in a fairly high percentage of Storey's (1933) operations the gut of the leafhopper was not actually damaged. Serial sections were made of insects 1, 2, 7, 8, and 12 days after the operation. Practically no healing was visible in the 1- and 2-day series, but in the 7- and 12-day series healing was apparent. In all these examples haemocytes were present at the site of the wound and had effectively closed it (Plate 2, Figs. 7 and 8). However, the bulk of the wound tissue was very small by comparison with *Periplaneta* and this was clearly due to the small number of haemocytes available. Haemocytes were never found to be involved in healing of cuticular wounds (Plate 2, Fig. 9). In distinction to the situation in *Periplaneta,* where mitoses were found in only one of over 100 examples of wound tissue studied, mitotic figures were found in every one of six examples of wound tissue in *Orosius.* This is undoubtedly correlated with the observation that haemocytes are too few to cover the wound completely and therefore undergo compensatory hypertrophy after having been incorporated in the wound tissue. A comparison was made of the role of haemocytes in walling off a small pellet of paraffin implanted in the abdomen of *Orosius.* Twenty-four hr after implantation in the haemocoele of *Periplaneta* such a pellet was covered by many layers of haemocytes (Day 1952, Plate 1, Fig. 5), but after a similar period no haemocytes were found surrounding a pellet in *Orosius.* Even 48 hr after implantation only a thin layer of haemocytes surrounded the pellet (Plate 2, Fig. 10).

Changes in the *Orosius* midgut epithelium were more difficult to follow than those of *Aedes.* There was no change in cell shape surrounding the wound shortly after puncture (compare Plate 2, Fig. 11, with Plate 1, Fig. 1), but in later stages of healing the epithelium was obviously involved (Plate 2, Figs. 8 and 12).

V. Discussion

It is clear from the observations described above that wound healing in insects can occur by mechanisms other than that described by previous authors (see Day 1952). In all previous reports the first and most conspicuous changes were produced by the haemocytes. These were responsible for the initial plugging of the wound and the formation of a bulky wound tissue. In *A. aegypti* wound healing can occur entirely without the intervention of haemocytes. Yet the process is as fast and as effective in the mosquito as in *Periplaneta.* In *O. argentatus* the haemocytes are responsible for the greater part of the healing process, but their numbers are inadequate and the process is slow because they must undergo mitosis at the site of the wound.

The eschar formed on the cuticle is the result of the exposure of haemolymph to the air, but the activities of the epithelium alone are sufficient to bring about midgut healing in *A. aegypti.* Haemolymph may play a part but this would not be visible in sections.

The rate of healing in *Aedes* and *Orosius* may be compared with that found in *Periplaneta.* In this cockroach, haemocytes begin to form effective wound tissue about 12 hr after the operation and the wound is generally fairly well covered after 24 hr. Epithelial growth is apparent after 3 days. In *Aedes* the wound is effectively closed after 24 hr, whereas in *Orosius* the wound is often still unhealed after 48 hr. Likewise encapsulation of a paraffin pellet is slower and less complete in *Orosius* than in *Periplaneta.*

The rate of healing is important from the viewpoint of the effect of wounding on the ability of the insects to transmit viruses. The data reported here suggest that exchange of substances may occur between the gut lumen and the haemocoele for over 24 hr without the necessity of penetrating epithelial cell membranes. The wound may be closed earlier than 24 hr or in rare instances may be plugged for more than 7 days with cells differing histologically from the normal midgut epithelium (Plate 1, Fig. 4). It seems likely that these regenerating cells differ in permeability from the normal epithelium, and so may permit the passage of materials to which they are normally impermeable. Once viruses such as eastern equine encephalomyelitis have reached the haemocoele they presumably find tissues satisfactory for their multiplication. The results of Philip (1948) suggest that this is not so for *Rickettsia burneti.* However, Weyer (1950, 1952) reported that several species of *Rickettsia* multiply in insect tissues other than those they normally invade if injected into the haemocoele.

The observations made in the course of this work have provided clear evidence for the existence of a membrane in *Aedes aegypti* surrounding the ingested blood cells. A peritrophic membrane has been generally thought to be absent in adult mosquitoes, although its presence has been claimed by Yaguzhinskaya (1940). Evidence will shortly be presented that the membranes observed in this work are, in fact, true peritrophic membranes (Waterhouse 1953).

VI. Acknowledgments

The authors wish to thank Mr. D. Wilson for his care with the photomicrographs, and Dr. D. F. Waterhouse for discussions concerning the mosquito peritrophic membrane.

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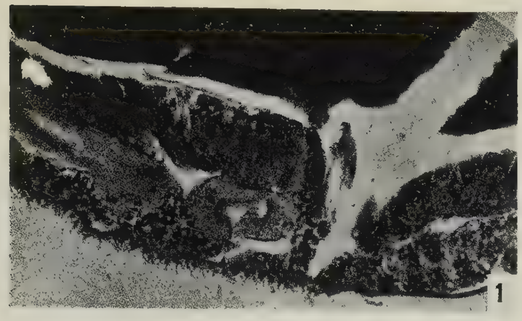
WOUND HEALING IN *AEDES* AND *OROSIUS* GUT



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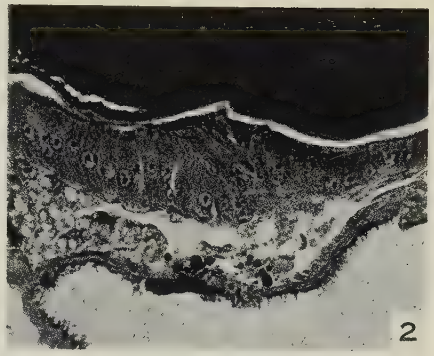

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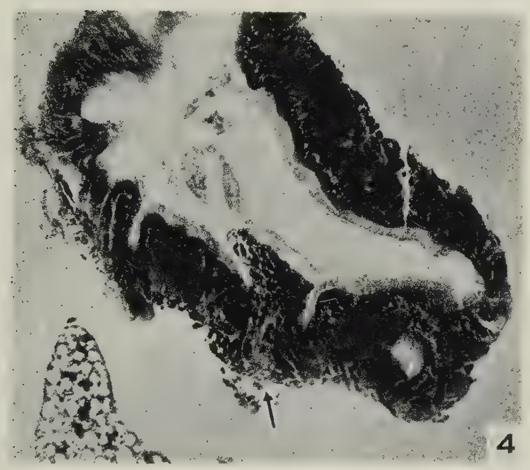

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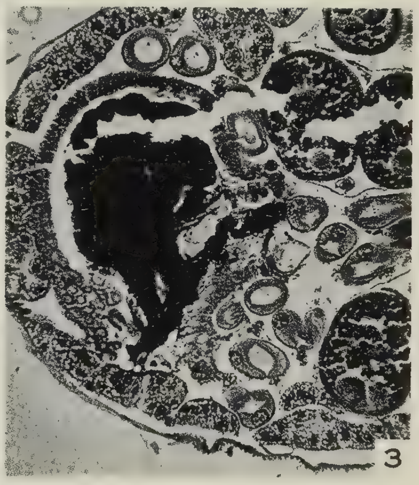

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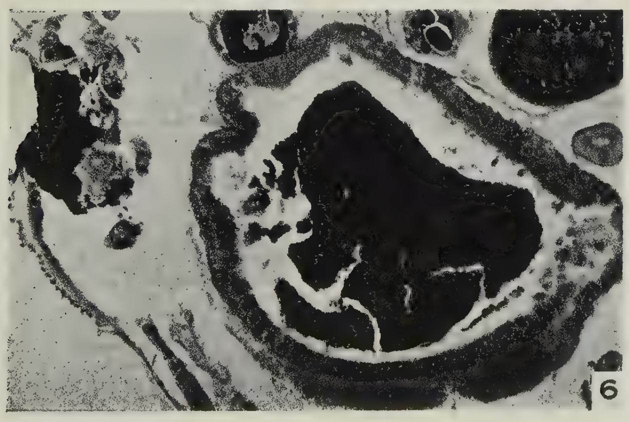

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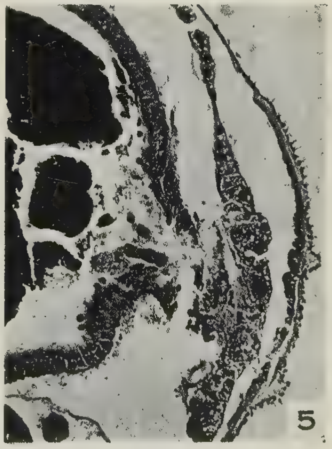

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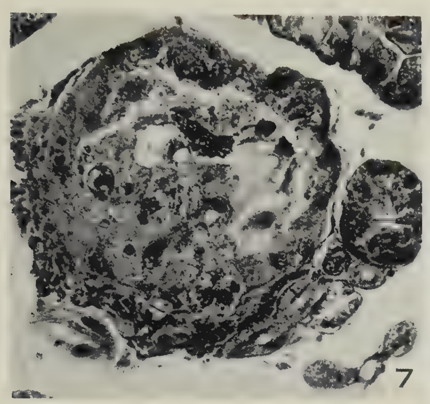
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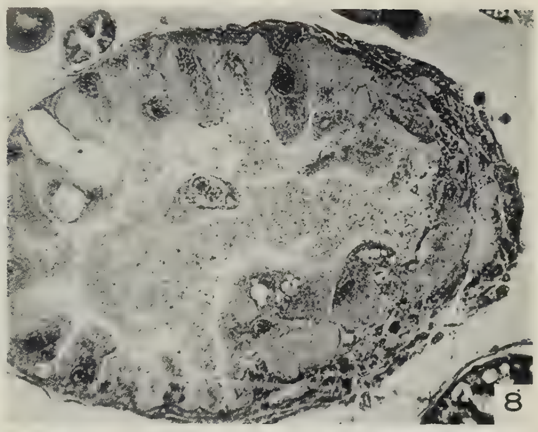
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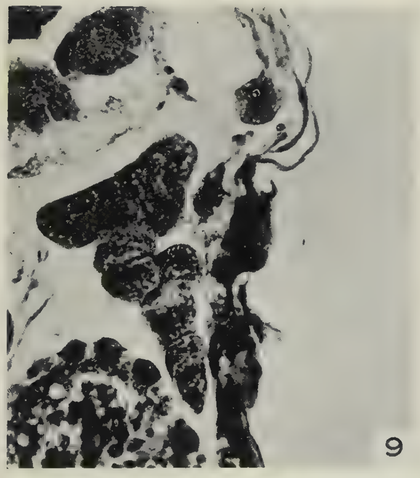
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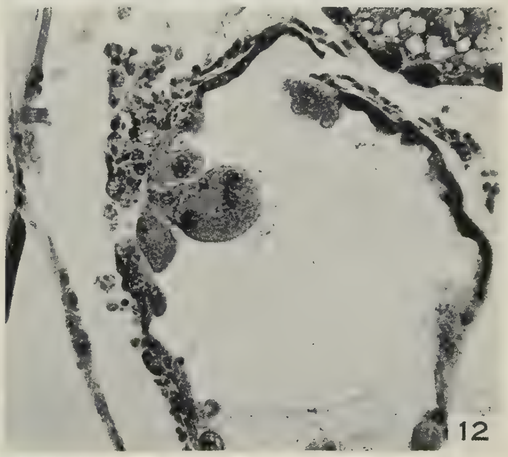
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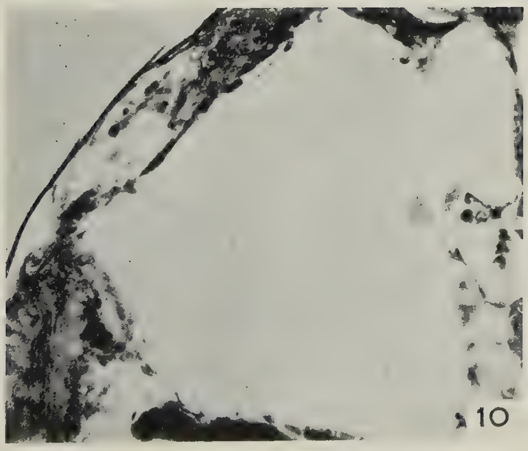
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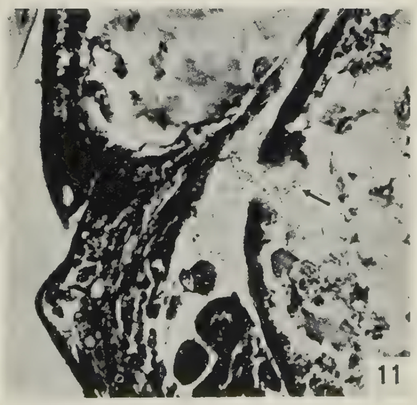
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EXPLANATION OF PLATES 1 AND 2

PLATE 1

Photomicrographs of 10-μ sections showing stages in wound healing in midgut of *Aedes aegypti.*

Fig. 1.—One hr after wounding. Note absence of epithelial regeneration; note differences in shape of epithelial cells surrounding the wound compared with those at the left hand top corner. The latter were typical of the cells in the remainder of the gut.

Fig. 2.—Sixteen hr after wounding. Midgut regeneration has resulted in closure of the wound. The cuticle wound and necrotic fat-body may also be seen.

Fig. 3.—One hr after wounding. The dark, partly digested erythrocytes have been carried by the needle into the wound. Epithelial cells in the vicinity of the wound are markedly vacuolated.

Fig. 4.—Seven days after wounding. Wound plugged by relatively unorganized group of epithelial cells at arrow. Note absence of haemocytes from wound tissue.

Fig. 5.—Fifty-four hr after wounding. Note absence of healing. Compare with Figure 6.

Fig. 6.—Almost complete regeneration of midgut epithelium 54 hr after wounding. Cuticular wound on the left has incorporated necrotic fat-body cells, but erythrocytes are entirely absent.

PLATE 2

Photomicrographs of 10-μ sections showing wound healing in *Orosius argentatus.*

Fig. 7.—Healing of midgut 7 days after wounding. Note that haemocytes have formed a wound tissue, but that necrotic epithelial cells are still present. Mitotic figures in the wound tissue were observed using oil-immersion objective.

Fig. 8.—Healing of midgut 8 days after wounding. Mitoses were present in wound tissue composed of haemocytes. Midgut epithelium more normal than in Figure 7.

Fig. 9.—Cuticular wound 7 days after wounding. Note complete absence of haemocytes. Eschar was intensely fuchsinophilic, and was apparently formed from coagulated haemolymph.

Fig. 10.—Loose wound tissue surrounding paraffin pellet implanted into abdomen for 48 hr. The pellet, now dissolved, occupies the large clear area. The slight role of the haemocytes in encapsulation is to be compared with their abundance in *Periplaneta* (see Day 1952, Plate 1, Fig. 5).

Fig. 11.—Midgut 2 days after wounding, showing absence of healing and flow of material at arrow from lumen into the haemocoele.

Fig. 12.—Midgut 12 days after wounding, showing sparse wound tissue in vicinity of wound. Regenerated epithelium has completely covered the injured midgut.

LOCALIZATION OF THE MAGNESIUM-ACTIVATED APYRASE OF INSECT MUSCLE IN THE SARCOSOMES

By D. Gilmour*

[*Manuscript received July* 6, 1953]

Summary

Sarcosomes of the fibrillar flight muscles of blowflies have been isolated in a reasonably pure state and shown to possess powerful apyrase activity (Q_p 2500-5000).

The evidence suggests that the Mg-activated apyrase of insect muscle previously described is located exclusively in the sarcosomes.

Only a negligible amount of apyrase activity appears in solution when the sarcosomes are suspended in a hypotonic medium.

The possible function of the apyrase is discussed.

I. Introduction

The Mg-activated ATP-ase of mammalian muscle, described by Kielley and Meyerhof (1948), has been shown by Perry (1952) to be associated with lipoprotein granules sedimenting at 14,000g, and has been related in function to the ATP-ase widely distributed in tissues, and associated with cytoplasmic granules. In insect muscle there is a Mg-activated apyrase which seems to correspond with the Kielley-Meyerhof enzyme (Gilmour and Calaby 1952). This apyrase is much more abundant in the indirect flight muscles than in other skeletal muscle. In considering the intracellular distribution of this enzyme, attention was directed to the sarcosomes, interfibrillar components of the muscle cells of insects, which have been shown to possess some of the enzymic properties of mitochondria (Watanabe and Williams 1951). The sarcosomes of insect muscle are most abundant and reach their largest size (up to 5 μ dia.) in the indirect flight muscles, particularly in the so-called "fibrillar" muscle of Diptera and Hymenoptera. Sarcosomes are present in the other skeletal muscles, but they are relatively few in number and of much smaller size. In this paper are described experiments which have shown that the Mg-activated apyrase is located in the sarcosomes.

Before the work reported here had been completed, the paper by Sacktor (1953) on the ATP-ase of the sarcosomes of *Musca* appeared. Sacktor demonstrated the presence in the sarcosomes of a Mg-activated ATP-ase, which, while not identified by him with the apyrase extracted from muscle, nevertheless had many of the properties of this enzyme. The present paper confirms and extends the observations of Sacktor.

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II. Experimental and Results

(*a*) *Apyrase and Phosphate Determinations*

The methods used in the determination of apyrase activity were the same as those described in the previous communication (Gilmour and Calaby 1952). Inorganic P was determined routinely by the Fiske and Subbarow (1925) technique. Since this method measures labile organic phosphate esters as inorganic P, and would thus mask the activity of enzymes transferring phosphate from ATP to an organic acceptor, the inorganic P determinations were checked by the Lowry and Lopez (1946) method. True inorganic P, by the Lowry and Lopez method, was approximately 90 per cent. of that measured by the Fiske and Subbarow technique. Figures for apyrase activity given have been corrected for this factor.

(*b*) *Fractionation of Blowfly Flight Muscle*

Sarcosome preparations were made from the fibrillar flight muscles of the adults of the blowflies *Calliphora stygia* (F.) and *C. fallax* Hardy, which were caught in traps at Canberra.

The thoracic muscles were dissected from a number of flies and dropped immediately into the ice-cold "Ringer" solution of Ephrussi and Beadle (1936), developed originally for use with *Drosophila* (NaCl 7.5 g, KCl 0.35 g, $CaCl_2$ 0.21 g per litre). The yield from 100 flies was about 1 g muscle. A glass rod was used to separate the muscle masses, breaking them up into small groups of fibrils, and releasing most of the sarcosomes. Gentle pressing and grinding against the side of the vessel was usually sufficient to effect this separation. The disintegrated muscle was then filtered through organdie with gentle pressure. The muscle fragments were resuspended and extracted twice more by this method. After the third extraction the volume of filtrate (S_1) was made up to 10 times the original weight of muscle. The muscle residue (R_1) was resuspended in Ringer solution (volume ≡ 15 times the original weight of muscle). Microscopic examination of the two fractions showed the filtrate to be a very dense and practically pure suspension of sarcosomes with no visible muscle fragments. The residue consisted mostly of separated muscle fibrils with numbers of sarcosomes still attached. The sarcosomes, removed from suspension in S_1 by centrifuging at 500g for 15 min, formed an orange-coloured plug at the bottom of the centrifuge tube. This residue (R_2) was resuspended in the original volume of Ringer solution. The supernatant from this separation (S_2) was then centrifuged at 20,000g for 15 min. The residue (R_3) was resuspended in the original volume of Ringer solution. The final supernatant (S_3) was clear. These manipulations were carried out at 0°C. Apyrase activities of the fractions obtained are shown in Table 1.

It is apparent that more than half the total apyrase activity was present in the sarcosome fraction, which contained approximately one-third of the total protein. A more complete separation was not possible, since a more vigorous treatment of the muscle broke up the fibrils into fragments which sedimented with the sarcosomes. Moreover, the actomyosin-ATP-ase of the fibrils probably contributed to the apyrase activity measured in R_1. Further centrifugations and resuspension of the sarcosome fraction revealed that 85-90 per cent. of the total activity was always associated with the particulate material sedimenting at 500g (see, for instance, Table 1, S_4 and R_4). Of the remaining 10-15 per cent. of activity, most remained in the supernatant even after centrifugation at 20,000g for 15 min. Thus, although a precise separation was not possible, it seems most likely that the Mg-activated apyrase activity was associated exclusively with the sarcosomes.

TABLE 1

FRACTIONATION OF BLOWFLY FLIGHT MUSCLE

Figures compounded from several experiments

Fraction	Weight of Protein per g Wet Weight Muscle (mg)	Total Apyrase Acitvity: Units (Q_P) per g Wet Weight Muscle
Muscle residue (R_1)	130	10,600
Muscle extract (S_1)	90	13,400
Residue after centrifuging S_1 at 500*g* for 15 min (R_2)	74	14,100
Supernatant after centrifuging S_1 at 500*g* for 15 min (S_2)	16	1,600
Residue after centrifuging S_2 at 20,000*g* for 15 min (R_3)	1	200
Supernatant after centrifuging S_2 at 20,000*g* for 15 min (S_3)	15	1,400
Residue after centrifuging R_2 (resuspended) at 500*g* for 15 min (R_4)	41	10,000
Supernatant after centrifuging R_2 (resuspended) at 500*g* for 15 min (S_4)	28	1,500

(*c*) *Experiments with Washed Sarcosomes*

By washing the sarcosomes several times with *Drosophila* Ringer solution, sedimenting at 500g on each occasion, it was possible to obtain a reasonably pure and active preparation, having a Q_p of from 2500 to 5000. The highest activities were always obtained by testing the preparation in 0.05M borate buffer at pH 8.0. In *Drosophila* Ringer solution, or in 0.16M NaCl, the activity was only about half as great, although the activity in Ringer could be increased by substituting Mg for Ca in the Ringer solution (Table 2). Substitution of Ca for Mg in the presence of borate buffer produced an almost complete inhibition, further confirming the identity of the sarcosome enzyme with the apyrase previously described (Gilmour and Calaby 1952).

It was thought that the higher activity in borate buffer might be accounted for by the rupture of the sarcosomes in hypotonic solution, followed by the release of apyrase into solution. It was observed through the microscope that sarcosomes in hypotonic solution tended to clump together, and that some were ruptured. Attempts to increase significantly the yield of apyrase in the supernatant by treating the sarcosomes with either water or 0.05M borate buffer, however, were unsuccessful. This is illustrated by some examples in Table 3. Of the small amount of apyrase removed from the sarcosomes by this treatment, most was associated with fragments sedimenting at 20,000g, i.e. the reverse of the result obtained in the first washings of the sarcosomes, as illustrated in Table 1.

TABLE 2

RELATIVE APYRASE ACTIVITIES OF VARIOUS PREPARATIONS OF WASHED SARCOSOMES

Suspending Medium	Activating Metal Added at Final Concentration of 0·001M	Activity, Expressed as Percentage of Maximum Activity
Borate buffer, 0·05M, pH 8·0	Mg	100
	Ca	12
Drosophila Ringer solution, pH 8·0	Mg	52
Drosophila Ringer solution with Mg substituted for Ca, pH 8·0	—	74
NaCl, 0·16M, pH 7·0	Mg	55

TABLE 3

REMOVAL OF APYRASE FROM SARCOSOMES BY SUSPENSION IN HYPOTONIC MEDIA

All activities measured in presence of 0·001M Mg and 0·05M borate buffer at pH 8·0, and expressed as a percentage of the activity of the original sarcosome suspension

Treatment	Specific Activity (per unit wt. protein)	Total Activity
Washed sarcosome suspension	100	100
Sarcosomes suspended 24 hr in water, centrifuged at 500*g* for 15 min; supernatant tested	60	12
Sarcosomes suspended 24 hr in water, centrifuged at 20,000*g* for 15 min; supernatant tested	30	1
Sarcosomes suspended 1 hr in 0·05M borate buffer, centrifuged at 20,000*g* for 15 min; supernatant tested	12	<0·1

Sacktor (1953) has attributed the hydrolysis of two phosphate groups of ATP by sarcosomes to the simultaneous action of ATP-ase and adenyl kinase. Although it is not doubted that an adenyl kinase is present, the apyrase is itself capable of dephosphorylating ADP (Gilmour and Calaby 1952) and sarcosomes hydrolyse IDP slowly (at about 7 per cent. of the rate of splitting of ATP) in spite of the inactivity of adenyl kinase against IDP.

III. DISCUSSION

It seems most likely that the sarcosomes, which in the highly developed flight muscles of Diptera and Hymenoptera comprise a significantly large proportion of the muscle protein, are concerned largely with the oxidative production of the energy needed to drive the muscle machine. Sarcosomes have been identified biochemically with mitochondria (Watanabe and Williams 1951) and, although so far it has not been possible to demonstrate oxidative phosphorylation in them (Sacktor 1953), their abundance in the flight muscles of insects fits in with the known rapid rate of oxidative metabolism during flight, and the very small oxygen debt of the flight muscles following exercise (Chadwick and Gilmour 1940; Krogh and Weis-Fogh 1951).

The role of the apyrase which is usually found associated with the oxidative enzyme systems in cell granules is not clear. It could act as a regulator, controlling the level of either ATP or inorganic P. Another possibility is that the enzyme is normally coupled with some other process, and that the destruction of ATP is an artefact induced by the separation procedure. It has been shown that an active ATP-ase can be extracted from liver mitochondria, which in the intact state have very little apyrase activity (Kielley and Kielley 1951, 1953). But the intact sarcosomes of insects, suspended in isotonic solution under apparently reasonably physiological conditions, still possess strong apyrase activity. If the apyrase of the sarcosome is coupled with any system to which the energy of the terminal phosphate of ATP can be transferred, it may not be unreasonable to look for this system outside the sarcosome rather than within it.

That the sarcosomes are in fact intimately concerned in the contraction in insect flight muscle can be deduced from the observations of Hanson (1952) on the ATP-contraction of isolated fibrils. Hanson found that contraction occurred only in fibrils to which some sarcosomes were adhering, and that when only a few sarcosomes were attached, the contraction wave could be seen to be starting at the point of attachment of the sarcosome. In other words, the unit of contraction was fibril plus sarcosomes. It is unlikely that the critical function of the sarcosomes was the production of phosphate bond energy, since ATP was present in excess. It may be that a more intimate role in the contraction process for the Mg-activated apyrase, which appears to be quantitatively the more important of the enzymes hydrolysing ATP in flight muscle (Gilmour and Calaby 1953), is to be found in this association of fibril and sarcosomes.

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THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY ERYTHROCYTIC STAGES OF *PLASMODIUM BERGHEI* (VINCKE & LIPS)

By P. R. Whitfeld[*]

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Summary

Radioactive phosphorus (^{32}P) was injected intraperitoneally into mice infected with *Plasmodium berghei* and the activity of the phosphorus-containing fractions of the parasites was determined at intervals during the subsequent 48 hr.

The lipid fraction had the highest content of ^{32}P; it contained half of the total ^{32}P present in the parasites. The ^{32}P content of desoxyribonucleic acid (DNA) was 3.5 per cent. of the total ^{32}P at the end of 2 hr, but increased to 24 per cent. of the total at the end of 48 hr. After 2 hr, the acid-soluble ^{32}P accounted for 23 per cent. of the total ^{32}P, but after 48 hr it accounted for only 13 per cent. At all times, 10 per cent. of the total ^{32}P was contained in the ribonucleic acid (RNA). The ^{32}P content of the phosphoprotein fraction was very low throughout the experiment.

The total ^{32}P content of the parasites after 48 hr was approximately four times the content after 2 hr.

I. Introduction

Most observations on the chemical properties of the erythrocytic stages of malaria parasites have been comparative in nature. The properties of the host's normal red blood cells have been compared with the properties of the infected red blood cells and any differences between them have been attributed to the parasites. Although much has been learnt by this method (Ball 1946; Ball *et al.* 1948), the results only indicate the apparent properties of the malaria parasite. No allowance can be made for the associated change in the properties of the red cells caused by the presence of the parasites.

Moreover, certain types of experiment by this technique are impracticable. For instance, in an experiment designed to show the incorporation of radioactive phosphorus into a malaria parasite, the activity of the host red blood cell would be so great, compared with that of the parasite, that the accuracy of the results must suffer.

The development of a quantitative procedure for the isolation of malaria parasites, uncontaminated with host material, has made possible the direct study of the chemical properties of these parasites. This paper describes the application of this technique to the examination of the uptake *in vivo* of radioactive phosphorus by *Plasmodium berghei.*

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II. Materials and Methods

The strain of *P. berghei* used in these experiments has been described previously (Whitfeld 1953). Blood was collected from infected mice by anaesthetizing them lightly and then severing the jugular vein. Powdered heparin was used as an anticoagulant.

Red blood-cell counts were carried out on a Spencer Bright Line hemacytometer and parasite counts were made on thin blood smears stained with Leishman's stain; enough cells were counted to reduce the error to less than 10 per cent. (Whitfeld 1953).

A modification of the procedure of Davidson, Frazer, and Hutchinson (1951) was followed for the separation of the various phosphorus-containing fractions of the parasites.

The agglutination of the red blood cells during the procedure for the isolation of the parasites was effected with "Intradex" (6.0 per cent. depolymerized "Dextran" in 0.9 per cent. sodium chloride) which was kindly supplied by The Crookes Laboratories, London.

Radioactive phosphorus was used as sodium dihydrogen phosphate in phosphate-saline (0.85 per cent. saline, 0.05M with respect to phosphate, pH 7.3). Radioactivity determinations were carried out on 16.0-ml samples in a liquid counter (Type M 12, 20th Century Electronics) attached to a scale of eight. Three 5-min counts, alternating with two 5-min background counts, were taken; this was sufficient to reduce the error to 5 per cent. in the lowest counts recorded, and to correspondingly less in higher counts. Corrections for ^{32}P decay were made when necessary. A standard solution of potassium carbonate (100 g per 100 ml) was used for testing variations in the sensitivity of the counter.

III. Procedure and Results

For each experiment 100 white mice were infected with *P. berghei* and blood films were made daily and examined. When approximately 20 per cent. of the red blood cells were parasitized, 4μc ^{32}P per 25 g body weight were injected intraperitoneally into each mouse.

Five minutes after the ^{32}P injection the blood was collected from three mice and centrifuged. A 0.5-ml sample of the plasma was taken and 1.0 ml ice-cold 10 per cent. trichloracetic acid was added. After 30 min at 0°C the precipitated protein was spun down and the supernatant set aside for the determination of the initial level of inorganic ^{32}P in the host plasma.

The remaining mice were divided into seven groups, each of which contained 12-14 mice. One group was killed after 2 hr, another after 4 hr, the others after 6, 8, 12, 24, and 48 hr, and for each group the pooled blood was centrifuged and the plasma removed. Of the plasma, 0.5 ml was kept for the determination of plasma inorganic ^{32}P. Two volumes of ice-cold phosphate-saline and 0.6 volumes of "Dextran" were added to the packed red cells, which were then resuspended and placed in the refrigerator. When the red cells had settled (20 min or less) the supernatant, which contained a few red cells and the majority of the leucocytes, was discarded. After resuspension in phosphate-saline the red cells were spun down lightly and the supernatant was discarded. A thin smear of the red cells was stained and examined immediately for leucocytes, and subsequently for the determination of the parasite count. If the number of leucocytes exceeded 0.01 per cent. of the red cells the cells were resuspended and the treatment with "Dextran" was repeated. Finally they were resuspended in saline to give a total volume of 10.0 ml and a red blood-cell count was carried out.

Of this supension, 2.0 ml was taken for total ^{32}P determination and the other 8.0 ml for separation into phosphorus-containing fractions. Both samples were hemolysed with saponin (Bovarnick, Lindsay, and Hellerman 1946) and the parasites freed from the ghost red blood cells by careful centrifugation. The parasites were washed with ice-cold saline till the washings showed no activity.

TABLE 1

ACTIVITY OF PLASMA INORGANIC PHOSPHORUS OF MICE INFECTED WITH *P. BERGHEI* AFTER INTRAPERITONEAL INJECTION OF 4μC ^{32}P/25 G BODY WEIGHT

Hours after ^{32}P Injection	0·1	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Activity (counts/min/ ml plasma)	30900	7720	3885	2340	2080	1860	1230	840

(*a*) *^{32}P Content of Mouse Plasma Inorganic Phosphate*

A suitable aliquot of the trichloracetic acid extract of the plasma was treated with acid molybdate in the presence of sodium chloride and extracted with butanol (Ennor and Stocken 1950). The activity of the butanol fraction was measured. The results are given in Table 1.

(*b*) *Total ^{32}P Content of* P. berghei

The parasites were ashed with a mixture of nitric, perchloric, and sulphuric acids (8:1:1 by volume). After neutralization the volume was made up to 16.0 ml and the activity determined (Table 2).

(*c*) *^{32}P Content of Phosphorus Fractions of* P. berghei

The parasites were extracted three times with ice-cold 10 per cent. trichloracetic acid; the extracts were combined and made up to a definite volume and the activity was measured. In the first experiment a fourth acid extraction was carried out and its activity determined. However, as this was found to be negligible, only three extractions were made in later experiments.

The residue was then extracted successively with acetone, alcohol, chloroform, and alcohol-ether (3:1) (twice) to remove the lipid fraction. The activity of the second alcohol-ether extract was determined separately and in every test was insignificant. After evaporation to dryness the lipid fraction was re-extracted with chloroform and the activity measured.

DNA (1.0 mg) was added to the acid-extracted, lipid-free residue, which was then digested overnight at 37°C in 1N KOH. The DNA was precipitated with acid-methanol and purified by redissolving in alkali and reprecipitating three times before it was finally dissolved and made up to 16.0 ml for determination of the ^{32}P content.

The supernatant, after precipitation of DNA, contained ribonucleotides and inorganic phosphorus which was derived from phosphoprotein. These two fractions were separated by butanol extraction in the presence of acid molybdate and sodium chloride. The activity of the butanol layer was then a measure of phosphoprotein ^{32}P and the activity of the aqueous layer was a measure of RNA ^{32}P.

TABLE 2

UPTAKE OF ^{32}P BY PHOSPHORUS FRACTIONS OF *P. BERGHEI**

Fraction	Hours After Injection of ^{32}P into Host						
	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Acid-soluble P	100	115	207	220	186	149	246
Lipid P	244	466	571	482	558	574	995
RNAP	45	68	56	70	98	115	187
DNAP	15	82	62	190	191	322	457
Phosphoprotein P	30	18	28	24	21	13	12
Total P (sum of fractions)	434	748	924	986	1054	1173	1897
Total P (experimental)	482	779	1009	1120	1196	1165	2086

* Activities are expressed as counts/min/10^{10} parasites.

Total ^{32}P content and ^{32}P content of the different fractions of *P. berghei* are given in Table 2. Results are expressed as counts per minute per 10^{10} parasites; each result is the average of at least two, but usually four, experiments.

It will be noted that at all times except in the 24-hr experiment the total ^{32}P content, calculated as the sum of the fractions, is less than that determined experimentally. This discrepancy, however, is not great and no doubt is due to small losses incurred during the long fractionation procedure.

The possibility of contaminating activity due to leucocytes in the parasite preparation was excluded by the following experiment. Five normal mice were each injected with 4 μc ^{32}P and their blood was collected 24 hr later. After removing the plasma, the cells were resuspended in phosphate-saline and "Dextran" was added. When the red cells had separated out the supernatant was discarded and the cells were washed once with saline and hemolysed. The ghost red blood cells were removed by careful centrifugation and the microscopical residue was washed with saline. The total ^{32}P content of this residue was negligible. It was concluded that the results given in Table 2 are a true indication of the ^{32}P content of the parasites.

An average dose of 4 μc ^{32}P per mouse might be considered larger than desirable but preliminary experiments had indicated that, unless a dose of this size was used, the activity of some of the fractions in the earlier stages was too low for measurement. An examination of stained preparations from dosed and non-dosed mice showed that neither parasite nor red blood-cell morphology was affected by the radioactivity.

IV. Discussion

It should be emphasized that the results given above are not expressed as specific activities. Thus, although the lipid fraction has the highest ^{32}P content, it does not necessarily follow that it has the highest specific activity. Similarly the ^{32}P content of the phosphoprotein is very low but its specific activity is probably quite high.

An examination of the variations with time in the relative amounts of ^{32}P in the different parasite fractions yields more information than an examination of the individual absolute ^{32}P contents at any particular time. These variations are emphasized if the ^{32}P content of the parasite fractions is expressed as a ratio of the host plasma inorganic ^{32}P (Table 3).

Table 3

RELATION BETWEEN ACTIVITY OF PHOSPHORUS FRACTIONS OF *P. BERGHEI* AND ACTIVITY OF HOST PLASMA INORGANIC PHOSPHORUS*

Fraction	Hours After Injection of ^{32}P into Host						
	2·0	4·0	6·0	8·0	12·0	24·0	48·0
Acid-soluble P	1·3	3·0	8·8	10·6	10·0	12·1	29·2
Lipid P	3·2	12·0	24·4	23·2	30·0	46·6	118·0
RNAP	0·6	1·7	2·4	3·3	5·3	9·3	22·2
DNAP	0·2	2·1	2·7	9·1	10·3	26·1	54·0
Phosphoprotein P	0·4	0·5	1·2	1·2	1·1	1·1	1·4
Total P (sum of fractions)	5·7	19·3	39·5	47·4	56·7	95·2	224·8

*Values are expressed as the ratio: $\frac{\text{activity of parasite fraction (counts/min/}10^{10}\text{ parasites)}}{\text{activity of host plasma inorganic P (counts/min/ml plasma)}} \times 100.$

Several interesting points become apparent. The lipid fraction has the highest ^{32}P content; it contributes approximately 50 per cent. of total ^{32}P and its value after 48 hr is 36 times its 2-hr value. On the other hand, the fraction in which the greatest increase in ^{32}P content occurs between 2 and 48 hr is the DNA; the 48-hr value of DNA ^{32}P is 270 times the 2-hr value and the contribution to total ^{32}P rises from 3.5 per cent. after 2 hr to 24 per cent. after 48 hr. As the ^{32}P content of RNA remains at about 10 per cent. of total ^{32}P during the 48-hr period, the ratio of RNA ^{32}P to DNA ^{32}P changes. Thus, after 2 hr the ratio is 3.0, but from 4 to 6 hr the ratio is 1.1 and after 8 hr it falls to 0.4.

The rate of incorporation of ^{32}P is faster during the early stages of the experiment. Between 24 and 48 hr the value of each fraction, except phosphoprotein, increases about 2.5-fold, whereas between 2 and 6 hr the values increase from fourfold (RNA) to 13-fold (DNA). Moreover, there seems to occur what might be termed a "lag-phase." From Table 3 it can be seen that there is very little increase in the values for the acid-soluble and DNA fractions during the period 8-12 hr, and for the lipid fraction during the period 6-8 hr. As schizogeny in *P. berghei* occurs between 6.00 a.m. and 10.00 a.m. (Ramakrishnan and Prakash 1951) and the experiments were always started about 9.00 a.m., then the period 6-12 hr later would correspond to the late trophozoite stage. Apart from this, there seems to be little point in attempting to correlate the changes in ^{32}P content of the fractions with some definite stage in the parasite's growth cycle.

Since this work was completed a paper has been published on the incorporation of ^{32}P into *Plasmodium gallinaceum* (Clarke 1952). In this paper the values are derived from differences between parasitized and non-parasitized red blood cells, but a comparison with the values presented above shows reasonable agreement between the two sets of results.

The possibility of a relationship between the mode of action of certain anti-malarials and parasite nucleic acids has been suggested by Parker and Irvin (1952), who demonstrated the binding power of chloroquine on DNA and RNA. An examination of the ^{32}P uptake by the nucleic acids of malaria parasites treated with chloroquine might shed some light on this problem. Furthermore, a comparison of the ^{32}P content in the nucleotides of nucleic acids isolated from normal and resistant strains of parasites is now possible and might lead to some interesting results.

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THE EFFECT OF pH AND CATIONS ON THE THERMAL DENATURATION OF TRYPSIN

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Summary

Dialysed or very dilute solutions of crystalline trypsin are rapidly and irreversibly inactivated at 40°C. At this temperature and in dilute solution the enzyme is most stable at pH values of about 2.7, 6.6, and 10.7, with intermediate regions of instability.

Between pH 3.0 and 6.5 all cations stabilize trypsin, salts of Cr, Al, Th, La, Fe+++, and Be affording considerable protection at 1×10^{-4}M, salts of Ca, Ba, Mg, and Mn at 1×10^{-2}M, and salts of K, Na, and NH_4 at 2M. Salts of Zn, Co, Ni, Cd, and Hg increase the stability of trypsin at 5×10^{-4}M but tend to inactivate the enzyme at higher concentrations. Between pH 7.0 and 9.5 where the most rapid inactivation takes place, cations soluble at the experimental pH stabilize the enzyme. The affinity of trypsin for cations increases with increasing pH.

Complexes of ferric salts with tartrate and pyruvate stabilize the enzyme at pH 8.0. Anionic ferritartrate is ineffective, stabilization being obtained only with the non-ionic complex which is formed in solutions of sufficiently low tartrate concentration.

On changing the conditions of inactivation to favour autodigestion of the enzyme, either by increasing the enzyme concentration or by heating for an extended period at 30°C, Mg was found to have little stabilizing effect whereas Ca was more effective than in dilute solutions of the enzyme at 40°C.

Evidence is presented that in dilute solutions of trypsin irreversible denaturation takes place at 40°C by a process which does not involve autodigestion or the formation of an intermediate reversibly denatured form of the enzyme.

I. Introduction

The earliest report of inactivation of trypsin by dilution was made by Rona and Kleinmann (1928) who showed that dilute solutions of crude trypsin lost up to 50 per cent. of their activity unless suitable concentrations of electrolytes were present. At pH values between 5.0 and 6.0 Al salts showed a considerable stabilizing effect at 0.003M, Mg salts at 0.01-0.05M, and Na salts at 2M.

Bier and Nord (1951) and Gorini (1951) showed crystalline trypsin to be unstable in solution at pH 8.0 and claimed that Ca and Mn salts were the only electrolytes providing significant protection against thermal inactivation.

There are also reports of "activation" of trypsin by metal ions. Michaelis and Stern (1931) found trypsin to be "activated" by certain coordinated Fe

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compounds such as trisdipyridyl iron (II) sulphate and "inhibited" by others such as ferritartrate. Kikuo Sugai (1944) reported that salts of Na, K, Mg, and Ca did not affect tryptic activity whereas $AlCl_3$ caused some increase in activity and salts of Mn, Fe, Co, and Ni caused marked increases in activity. Bresler and Rosetzveig (1951) found dialysed preparations of crystalline trypsin to be "activated" by salts of Mg and Cr and "inhibited" by metal-chelating agents. The "inhibition" of commercial preparations of the enzyme by such reagents was ascribed to their reaction with Cr.

The data in many of these papers are not sufficient to distinguish between activation and stabilization by metal ions during the preliminary warming up and subsequent incubation. Changes in pH of the enzyme solution on addition of salts before incubation of the enzyme with the substrate were not checked, and the course of breakdown of the substrate in the presence and in the absence of metal ions was not investigated. This paper describes the influence of pH, temperature, cations in the presence and absence of buffers, and coordinated metal complexes on the activity and stability of trypsin, and reports some preliminary kinetic experiments on the thermal inactivation of trypsin.

II. Materials and Methods

Crystalline trypsin (Armour) was used in most experiments and some crucial experiments were repeated with a crystalline preparation kindly supplied by Dr. Elizabeth Work.

Apart from $Th(NO_3)_4$ and $BeCl_2$, which were of "Laboratory Reagent" quality, all chemicals were of "Analar" quality. Except for Th, which was added to the enzyme as the nitrate, chlorides were used in all experiments.

The activity of the trypsin solutions was measured by the gravimetric method for determining gelatin hydrolysis (Crewther 1952) and in some experiments by the haemoglobin digestion method of Anson (1938). The gelatin used for preparing buffer substrate solution for the gravimetric method contained considerable amounts of Ca salts, which could be readily demonstrated by precipitation with oxalate. Unless otherwise specified, digestions were carried out at 40°C and pH 7.0, the optimum pH at that temperature as estimated by both methods. In experiments using the gravimetric method, thermal inactivation of the enzyme was carried out by pipetting 0.1 ml of a well-dialysed solution containing approx. 1 mg crystalline trypsin per ml into 20 ml of a solution of the electrolyte standing in a water-bath at the required temperature. The solution was immediately shaken and returned to the water-bath for the experimental period before estimating residual activity. When the haemoglobin digestion method was used to estimate residual activity the concentration of the enzyme was increased tenfold and in some experiments only 10 ml of the electrolyte solution was used. Dialysis of a solution containing 10 mg/ml of the enzyme was carried out through Visking cellulose tubing in distilled water without agitation for 2-3 days at 0-3°C. The solution was then diluted with distilled water if necessary. A glass electrode assembly was used to measure pH.

III. EXPERIMENTAL

(a) *Effects of Dilution and Dialysis of Trypsin Solution*

Figure 1 shows the activities of a series of dilutions of a trypsin solution using distilled water as diluent, and of similar solutions diluted with the boiled diffusate from a concentrated solution of crude trypsin. At low enzyme concentrations there was considerable inactivation in the solution diluted with water. Dialysis of solutions of crystalline trypsin against running tap water* for several hours resulted in almost complete inactivation, and activity was not restored by addition of salts of K, Na, Mg, Ca, Mn, Fe, Co, or Cu, or by the addition of diffusate from crude trypsin solutions (Table 1). However, dialysis against salts of any of the above metals or dialysis in the cold caused considerably less diminution in activity (Table 1). Similarly the use of various electrolyte solutions for dilution of trypsin solutions reduced the inactivation at high dilution.

TABLE 1

EFFECTS OF DIALYSIS AND METAL IONS ON THE STABILITY OF TRYPSIN

Trypsin Solution	Additions	Activity (gravimetric units)
Undialysed	Nil	12·6
Dialysed against distilled water, 24 hr at room temperature	Nil	0·5
	$MgSO_4$ 0·01M	0·6
	$CaCl_2$ 0·01M	0·7
	$CoCl_2$ 0·01M	0·4
	$MnCl_2$ 0·01M	0·5
Dialysed against boiled diffusate from crude trypsin, 24 hr	Nil	9·7
Dialysed against 0·001M $MgSO_4$	Nil	9·7
Dialysed against 0·1M NaCl	Nil	8·5
Dialysed against distilled water, 48 hr at 2°C	Nil	11·8
Dialysed at 2°C, held at 40°C 1 hr, pH 7·8	Nil	0·3
pH 8·4	$MgSO_4$* 0·001M	9·2
pH 8·1	$CaCl_2$* 0·003M	10·2
pH 7·6	$MnCl_2$* 0·003M	9·3
pH 9·4	KCl* 1M	8·0
pH 7·5	$Al_2(SO_4)_3$* $<0·0001$M	3·4

* Added before heating the enzyme to 40°C.

(b) *Relation between pH and Stability of Trypsin Solutions*

A dialysed solution of trypsin was diluted 1/200 with dilute HCl or NaOH at 40°C to give a suitable range of final pH values, and held at that temperature in stoppered tubes. Residual activity was measured after 60 min and the final pH values determined immediately. There were three peaks of maximum stability at pH approx. 2.9, 6.6, and 10.7 (Fig. 2) and inflexions in the curve at pH 3.5 and 6.0. The activity values at the extreme acid end of the curve

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* The tap water in Melbourne contains approx. 5 p.p.m. Ca and may reach a temperature of 21°C in summer.

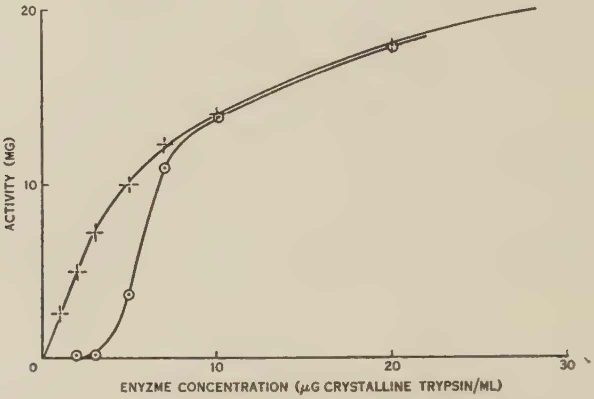

Fig. 1.—Inactivation of trypsin by dilution. ⊙, Trypsin diluted with distilled water. +, Trypsin diluted with diffusate from crude trypsin solution.

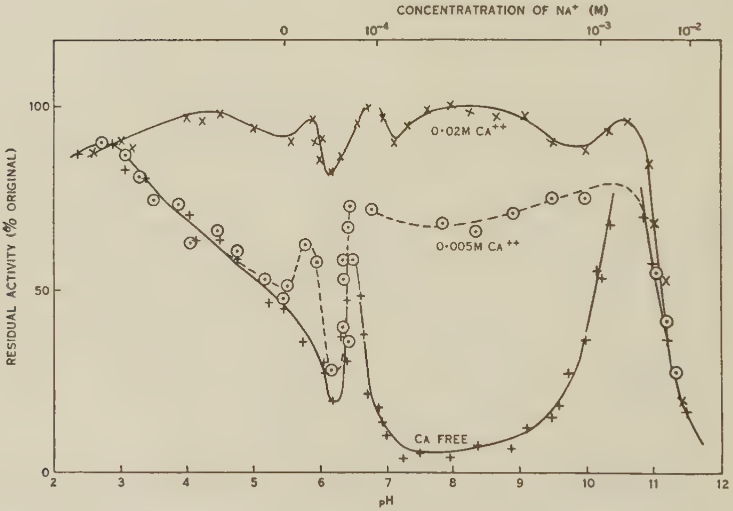

Fig. 2.—Effect of pH on the stability of trypsin in the presence and absence of calcium salts. +, pH-stability curve in the absence of added cations. ⊙, pH-stability curve in 0.005M CaCl$_2$. ×, pH-stability curve in 0.02M CaCl$_2$. The ordinates are the residual activities after holding 30 min at 40°C.

were slightly low, owing to a small reduction in pH of the buffer substrate on adding the enzyme solution. The accurate estimation of pH in the final unbuffered enzyme solutions was made difficult by solution of CO_2 and by the slow rate at which the glass electrode came to equilibrium with the solution in the absence of buffer. Errors of ± 0.1 pH unit probably occurred. A similar relationship between trypsin stability and pH was obtained using the haemoglobin digestion method for estimating residual activity.

(*c*) *pH-Activity Curves*

Under the conditions usually employed for estimation of tryptic activity (26°C for 10 min (Anson 1938)), a pH optimum of 7.5 is obtained. With a digestion temperature of 40°C the gravimetric technique provided the curves shown in Figure 3, the optimum activity being at pH *c.* 7.0 with a subsidiary peak at *c.* 9.0. To ensure that a single enzyme was being estimated, the pH-activity curve of a solution of trypsin was compared with that of the same solution after partial heat inactivation at pH 8.0. The ratio of activities over the whole pH range was constant within experimental error (Fig. 3). This suggests that the dual peaks were due to inactivation of the enzyme during incubation with the substrate at 40°C between pH 7.0 and 9.0, and not to the presence of more than one enzyme. The incorporation of 0.005M $CaCl_2$ in the buffer substrate solution or reduction of the temperature of incubation to 25°C provided curves with a single peak at a pH of approx. 7.6. When Anson's haemoglobin method was used at 40°C to determine the pH-activity curve, a single activity peak was obtained at pH 7.0. The differences in the pH-activity curves obtained by the gravimetric method and the haemoglobin digestion method are attributable to the greater amount of inactivation of the enzyme during the longer incubation period of the gravimetric method.

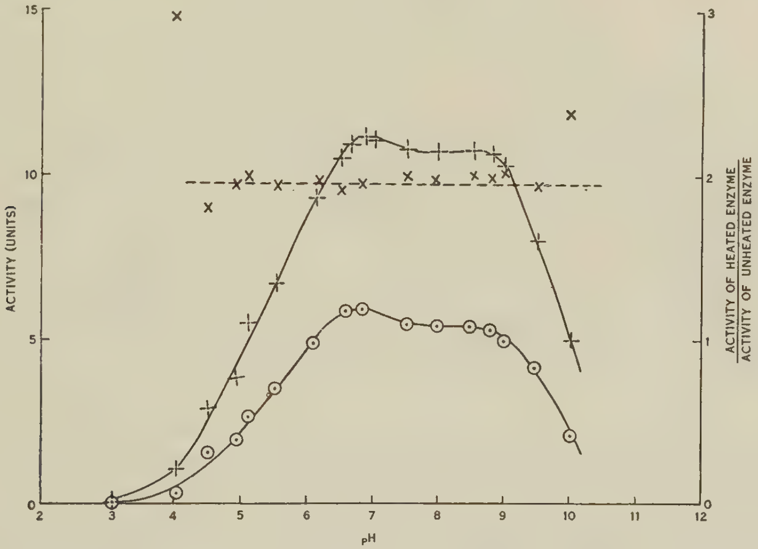

Fig. 3.—pH-activity curves of trypsin and partially inactivated trypsin. +, Native trypsin. ⊙, Trypsin partially inactivated at 40°C. ×, (Activity native trypsin)/(Activity partially inactivated trypsin).

(*d*) *Stabilization of Trypsin by Cations*

It was difficult to ensure that a predetermined pH would be obtained on mixing dialysed enzyme solution with unbuffered salt solutions, particularly between pH 7.0 and 9.0. By pre-testing mixtures of enzyme and the salt solutions the range of final pH values for a series of dilutions of a particular salt could be limited sufficiently for practical purposes, but considerable variations in pH occurred from one salt to another.

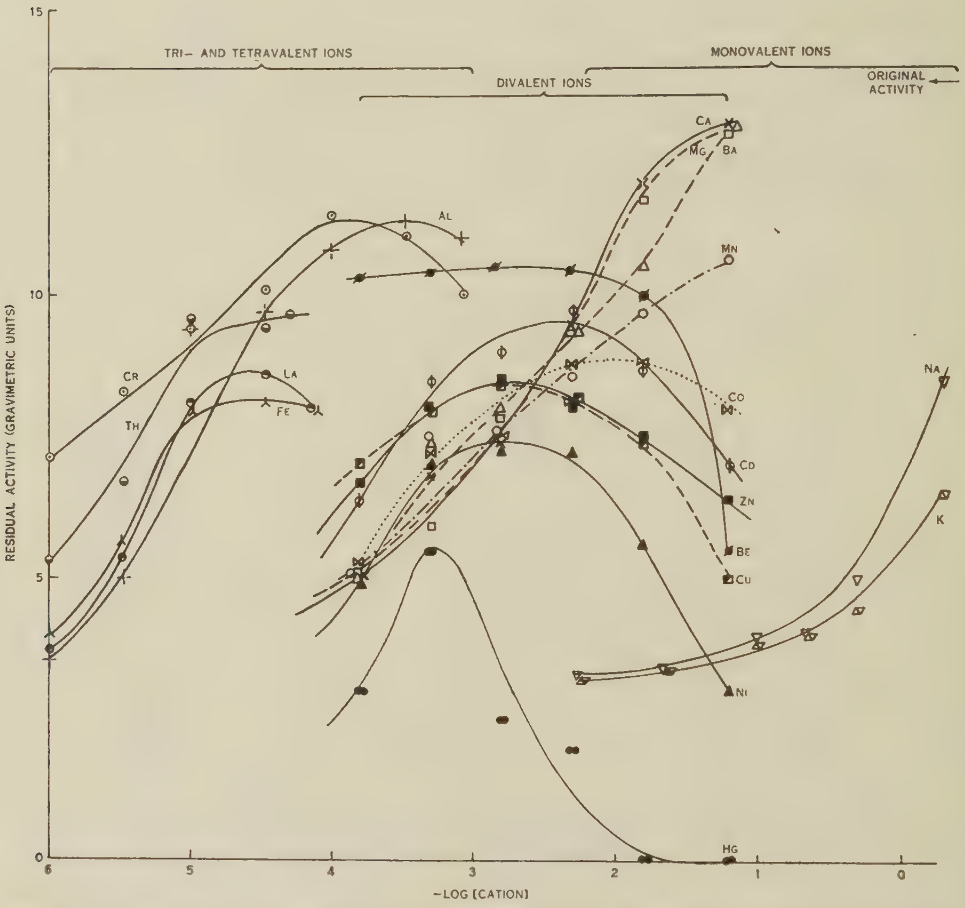

Fig. 4.—Stabilization of trypsin against treatment for 1 hr at 40°C in the presence of metal chlorides. The pH ranges for the various cations were:

Th (as the nitrate) 5.05 ± 0.10
Cr 6.0 ± 0.25
Al 6.20 ± 0.25
La 4.91 ± 0.06
Fe 4.95 ± 0.08
Be 6.00 ± 0.15

Ca 6.10 ± 0.15
Mg 6.05 ± 0.15
Ba 5.95 ± 0.15
Mn 5.9 ± 0.30
Co 6.0 ± 0.40
Cd 6.90 ± 0.20

Zn 5.80 ± 0.15
Cu 6.62 ± 0.10
Ni 6.10 ± 0.20
Hg 5.33 ± 0.25
Na 6.05 ± 0.10
K 6.12 ± 0.15

Figure 4 shows the residual activity after 1 hr at 40°C of trypsin solutions containing various concentrations of salts at pH values between 4.9 and 6.2.

Salts of Th, Cr, Al, La, Fe^{+++}, and Be stabilized the enzyme to a considerable degree at concentrations of 0.0001M whereas most divalent cations gave maximum stabilizing effects at 0.0005M to greater than 0.05M. It will be seen that, in general, the divalent cations providing the greatest degree of stability at concentrations of 0.005M caused the greatest inactivation or inhibition of the enzyme at 0.05M. The monovalent cations showed little effect at concentrations below 0.5M, and even at 2M preserved only 45-60 per cent. of the original activity. In the pH range 7.0-9.0 Mg, Co, and NH_4 stabilize trypsin (Fig. 5); Table 1 provides data for other cations in this pH range.

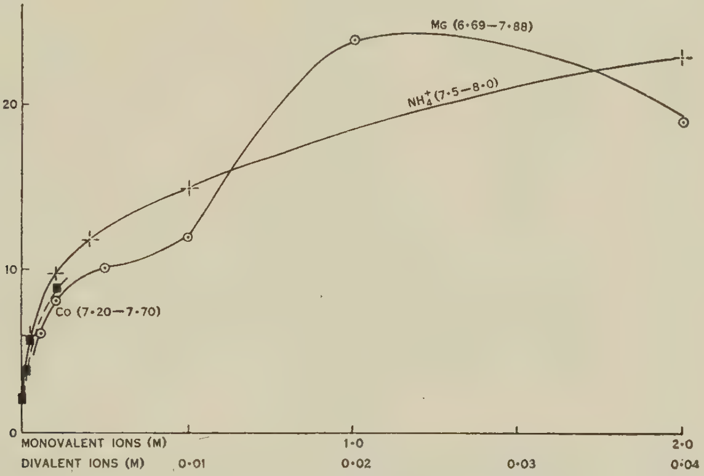

Fig. 5.—Comparison of the stabilizing effects of cations (40°C for 1 hr) in the pH range 7.0-9.0. pH ranges of the final solutions are indicated in brackets.

In view of the results in Figure 4 and the findings of Gorini (1951) and Bier and Nord (1951) that Ca^{++} and Mn^{++} are the only cations which stabilize trypsin at pH 8.0, a further comparison of the stabilizing effects of Ca^{++} and Mg^{++} was made in unbuffered solutions, with particular attention to pH control. Figure 6 shows $CaCl_2$ and $MgCl_2$ to have equal stabilizing effects at pH 6.9 ± 0.05.

Ca^{++} and Mg^{++} were also tested for stabilization at pH 7.9 in solutions containing 0.1M borate buffer and in 0.1M NaCl solution (this being the approximate Na^+ concentration of the buffer). Control solutions containing no cations other than those added in pH adjustment, and buffer solution and NaCl solution containing no added cations were also tested for stabilization of the enzyme. With each of the unbuffered solutions a series of tests was conducted to give final pH values on either side of 7.9, and the residual activity at pH 7.9 was estimated graphically. In solutions containing NaCl, Ca^{++} was slightly more effective than Mg^{++}, whereas in borate the order was reversed (Table 2), possibly owing to the greater tendency for Ca^{++} to form a complex with borate. The stabilizing effects of the Na^+ of the borate and NaCl solutions were also apparent. A similar experiment using higher concentrations of the enzyme, and using the haemoglobin digestion method for estimating trypsin activity, is summarized in Table 3. In this experiment the undoubted stabilizing effects of Mg^{++} were masked by the comparable effects of the buffer. However, under the conditions of this experiment Mg^{++} was inferior to Ca^{++} whether in borate, in NaCl solution, or in the absence of other electrolytes.

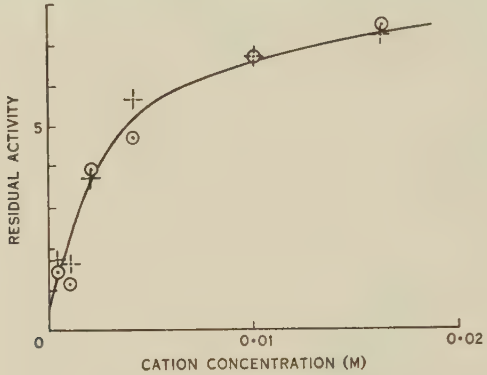

Fig. 6.—Comparison of the stabilizing effects of calcium and magnesium ions at pH 6.9 ± 0.05. Trypsin solutions held 1 hr at 40°C. ⊙, Ca^{++}; +, Mg^{++}.

Table 2

STABILIZATION OF TRYPSIN SOLUTIONS BY 0·001M $CaCl_2$ AND $MgCl_2$ IN THE PRESENCE OF 0·1M BORATE BUFFER OR 0·1M NaCl AT pH 7·9

Trypsin solutions held at 40°C for 30 min

Buffer (0·1M)	Cation Added (0·001M)	Residual Gravimetric Activity (% original activity)
Borate	Nil	36
Borate	Mg	61
Borate	Ca	60
NaCl	Nil	49
NaCl	Mg	63
NaCl	Ca	71
Nil	Na	7

That these effects are due to a real stabilization of the enzyme and not to a secondary effect of the cation on the method of estimating residual activity was demonstrated as follows: An enzyme solution of twice the normal concentration was pipetted into salt solutions and water controls at 40°C. After 1 hr the salt solutions were diluted with an equal volume of ice-water and the control solutions with an equal volume of chilled salt solution; the activity of each solution was then determined. Table 4 shows that trypsin solutions which contained 0.005M $MgCl_2$ at pH 5.9, or 2M KCl at pH 6.7 during heating at 40°, retained a much greater proportion of the original activity than enzyme solutions at the same pH to which the salts were added after heating.

TABLE 3

STABILIZATION OF TRYPSIN BY CALCIUM AND MAGNESIUM IN ABSENCE AND PRESENCE OF BORATE BUFFER, AS DETERMINED BY THE DIGESTION OF HAEMOGLOBIN

Dialysed solution (0·1 ml) of crystalline trypsin containing 10 mg/ml pipetted into 10 ml of electrolyte solution at 40°C and held 1 hr at pH 8·3

Buffer	Metal Ion	Residual Acitivty (trypsin units)
Nil	Nil	0·6*
Nil	Ca 0·005M	5·8*
Nil	Ca 0·0005M	5·7*
Nil	Ca 0·00005M	1·0*
Nil	Mg 0·005M	5·2*
Nil	Mg 0·0005M	3·1*
Nil	Mg 0·00005M	0·9*
Borate 0·1M	Nil	6·4
Borate 0·1M	Ca 0·005M	11·0
Borate 0·1M	Ca 0·0005M	8·1
Borate 0·1M	Ca 0·00005M	6·2
Borate 0·1M	Mg 0·005M	7·9
Borate 0·1M	Mg 0·0005M	6·0
Borate 0·1M	Mg 0·00005M	6·2
NaCl 0·1M	Nil	6·3*
NaCl 0·1M	Ca 0·005M	10·5*
NaCl 0·1M	Ca 0·0005M	7·3*
NaCl 0·1M	Ca 0·00005M	6·1*
NaCl 0·1M	Mg 0·005M	6·8*
NaCl 0·1M	Mg 0·0005M	5·6*
NaCl 0·1M	Mg 0·00005M	5·5*

* Activities at pH 8·3 determined graphically from a series of determinations.

(e) *Effect of pH on Stabilization by Cations*

Curves relating pH and the residual activity after heating 1 hr at 40°C were obtained for trypsin solutions containing various concentrations of $CaCl_2$. Figure 2 shows that, in the pH range 7.0-10.5, Ca stabilized the enzyme at a lower concentration than in the pH range 3.0-6.5. Similarly 0.1M Na stabilized trypsin to a greater extent at pH 7.9 than at 6.0 (cf. Fig. 4 and Table 2).

(f) *Reaction of Cations with Trypsin and their Role in the Digestion of Proteins*

A 2 per cent. gelatin substrate solution containing the usual amounts of glacial acetic acid and NaOH to give a pH of 8.0 was prepared from gelatin which had been freed of cations by passage through a column of "Amberlite

IR120." No alkaline earth cations could be detected by precipitation as oxalates. Four solutions containing 20-ml aliquots of the buffer-substrate with 1 ml of a dialysed solution containing 0.1 mg/ml crystalline trypsin and 4 ml of water or of a 0.02M solution of $CaCl_2$, $MgCl_2$, or $MnCl_2$ at pH 8.0 were incubated at 40°C. The progress of digestion in each solution was followed at intervals by the gravimetric technique using 2.5-ml samples. Figure 7 shows that the initial rate of digestion was independent of the presence of cations and demonstrates a considerable progressive inactivation of the protease during incubation with a substrate solution containing no cations but Na^+.

Table 4

STABILIZATION OF TRYPSIN BY 2M KCl AT pH 6·5 AND BY 0·005M $MgCl_2$ AT 5·9 WHEN HEATED AT 40°C FOR 1 HR

Final trypsin solutions of both control and test solutions contained either 1M KCl or 0·0025M $MgCl_2$

Electrolyte Present During Heating	Final pH	Residual Activity (units)
KCl 2M	6·7	7·9
NaOH approx. 10^{-4}M	6·7	1·0
	6·8	0·9
$MgCl_2$ 0·005M	5·9	5·8
NaOH approx. 10^{-4}M	6·0	2·6
Enzyme not heated		9·5

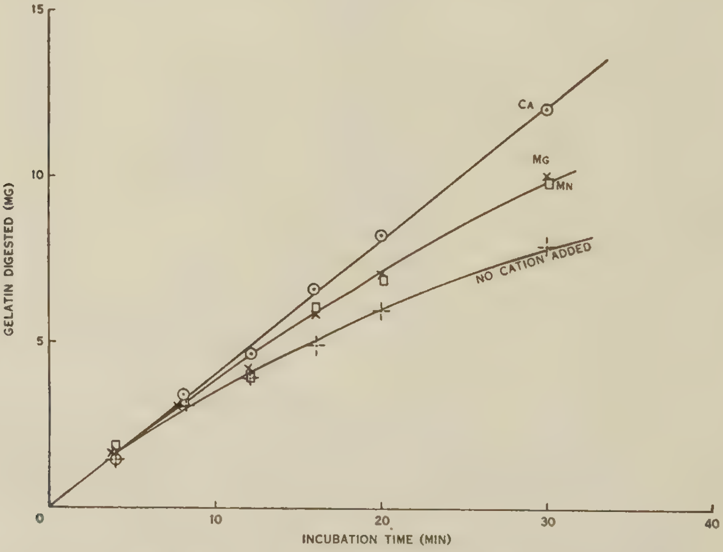

Fig. 7.—Relationship between digestion of gelatin by trypsin at 40°C and time, in the presence and absence of cations. ⊙, Ca++; ×, Mg++; □, Mn++; +, no cation.

The reaction between enzyme and cations was very rapid. Thus addition of $CaCl_2$ to the enzyme solution before mixing with a solution of $CaCl_2$ of the same concentration at 40°C and pH 8.0 did not affect the rate of inactivation.

TABLE 5

EFFECT OF TRYPSIN CONCENTRATION ON ITS RATE OF INACTIVATION AT 40 AND 30°C

Enzyme heated in 0·02M borate buffer at pH 8·0 with and without addition of calcium or magnesium salts, 40°C for 1 hr or 30°C for 24 hr

Cation Added	Concentration of Trypsin (mg/ml)	Heat Treatment	Dilution Before Estimation	Residual Activity (gravimetric units)
Nil	0·6	1 Hr at 40°	1/100	0·6
Ca 0·002M	0·6	1 Hr at 40°	1/100	4·9
Ca 0·005M	0·6	1 Hr at 40°	1/100	5·1
Mg 0·002M	0·6	1 Hr at 40°	1/100	0·6
Mg 0·005M	0·6	1 Hr at 40°	1/100	0·8
Nil	0·06	1 Hr at 40°	1/10	1·0
Ca 0·002M	0·06	1 Hr at 40°	1/10	3·3
Ca 0·005M	0·06	1 Hr at 40°	1/10	4·0
Mg 0·002M	0·06	1 Hr at 40°	1/10	1·6
Mg 0·005M	0·06	1 Hr at 40°	1/10	3·5
Nil	0·006	1 Hr at 40°	1/1	0·5
Ca 0·002M	0·006	1 Hr at 40°	1/1	2·3
Ca 0·005M	0·006	1 Hr at 40°	1/1	2·9
Mg 0·002M	0·006	1 Hr at 40°	1/1	1·8
Mg 0·005M	0·006	1 Hr at 40°	1/1	3·7
Ca 0·001M+Mg 0·001M	0·006	1 Hr at 40°	1/1	2·0
Ca 0·0025M+ Mg 0·0025M	0·006	1 Hr at 40°	1/1	3·6
Nil	0·6	24 Hr at 30°	1/100	0·4
Ca 0·005M	0·6	24 Hr at 30°	1/100	6·3
Mg 0·005M	0·6	24 Hr at 30°	1/100	1·7
Nil	0·006	24 Hr at 30°	1/1	0·7
Ca 0·005M	0·006	24 Hr at 30°	1/1	5·6
Mg 0·005M	0·006	24 Hr at 30°	1/1	2·0

(g) *Effect of Trypsin Concentration on Stabilization by Cations*

Three different concentrations of trypsin in 0.1M borate buffer at pH 8.0 were heat-treated in the presence and in the absence of $CaCl_2$ or $MgCl_2$, diluted to the concentration of the most dilute solution with ice-cold 0.001N HCl, and residual activities measured. Table 5 shows that at high trypsin concentrations Ca^{++} was a more effective stabilizing agent than at low enzyme concentrations, whereas Mg^{++} had relatively little effect at high enzyme concentrations but was as effective as Ca^{++} at low enzyme concentrations. With lower temperatures of inactivation and greater time of heating, Ca^{++} was more effective than Mg^{++} at each trypsin concentration tested.

(*h*) *Stabilization of Trypsin with Coordinated Ferric Complexes*

Solutions of 0.0017M $FeCl_3$ containing various concentrations of tartaric or pyruvic acids were compared for stabilizing effect at pH 8.0 with similar solutions of the same acids containing 0.005M NaCl (approximating the concentration of NaOH required to neutralize the $FeCl_3$) in place of the $FeCl_3$ salt. The iron-containing solutions with the lower concentrations of the organic acids contained precipitates of basic salts which were filtered off. Tartrate interfered with the gravimetric protease method by co-precipitating with the gelatin; the haemoglobin digestion method was therefore used. Table 6 demonstrates the considerable stabilizing effects of the Fe coordination complexes as compared

TABLE 6

STABILIZATION OF TRYPSIN BY COORDINATION COMPLEXES OF FERRIC IRON WITH TARTRATE AND PYRUVATE AT pH APPROX. 8·0

Enzyme-complex mixture heated at 40°C for 30 min

Stabilizing Solution	Final pH	Residual Activity	
		Gravimetric Method (units)	Anson's Method* (units × 10^4)
$FeCl_3$ 0·003M +			
tartrate 0·1M (yellow)	8·3		1·9
0·05M (yellow)	7·8		1·9
0·025M (yellow)	7·3		2·0
0·012M (orange)	6·8		6·1
0·003M (orange)	7·1		5·0
pyruvate 0·1M	8·1		1·5
0·05M	7·8		1·8
0·025M	7·4		3·6
0·012M	7·8	7·3 (pH 8·9)	4·5
0·003M	8·1		2·8
NaCl (0·01M) +			
tartrate 0·1M	7·0		1·6
0·05M	7·0		1·7
0·025M	7·1		2·0
0·012M	6·8		2·7
0·003M	7·1		2·0
pyruvate 0·1M	7·3		2·0
0·05M	7·0		1·1
0·025M	7·3		—
0·012M	7·8	1·3 (pH 8·7)	0·8
0·003M	7·6		0·7
Fe^{+++} saturated at pH 6·8	6·5	2·0	—
HCl (0·001N) (enzyme not heated)	2·8	9·4	7·3

* As the experimental conditions were not standard these units are not identical with standard trypsin units.

with Fe salts at the same pH, or with Na^+ ions at the concentration present in the complex solutions. Stabilization occurred chiefly in the solutions containing a low concentration of the ligand. With higher concentrations of the ligand the stabilization could be attributed to the Na^+ ions in the solution introduced during pH adjustment.

Filter-paper ionophoresis of the complex solutions containing 0.1M tartrate and 0.003M tartrate was carried out in tartrate of the same concentration as the test solution at pH 8.0 with a potential gradient of 2.5 V/cm for 1 hr, the paper then being sprayed with thiocyanate. In the lemon-yellow solution containing 0.1M tartrate the iron was anionic and in the solution containing 0.003M tartrate it was non-ionic.

(*i*) *Kinetic Experiments*

The progress of trypsin inactivation with time was followed at various pH values. There were slight differences in the form of the curves obtained with buffered and unbuffered solutions which may be due to changes in the pH of the solutions during denaturation. The results of the experiments in which buffers were used are shown in Figures 8 and 9.

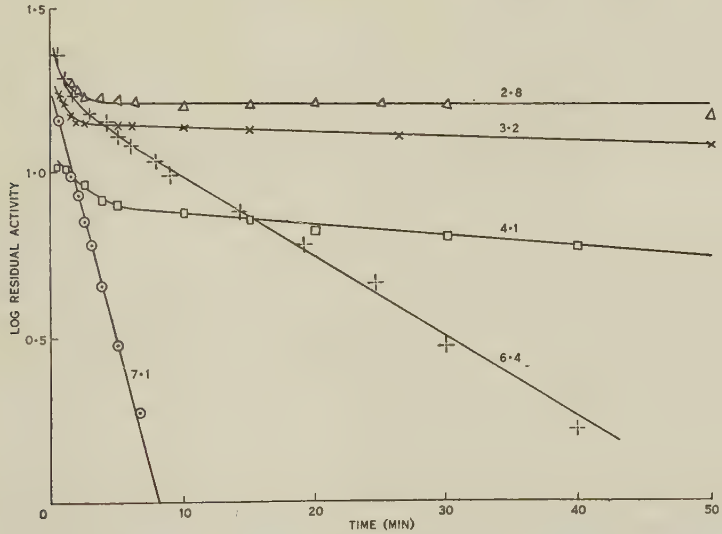

Fig. 8.—Kinetics of inactivation of trypsin at 40°C. pH values are indicated on the curves. Buffers were 0.01M formate (pH < 4.0) and acetate (pH 4.0-6.5).

IV. Discussion

The pH-stability curve obtained by Kunitz and Northrop (1934) for crystalline trypsin, after holding at 30°C for 24 hr, shows optimum stability at pH 2.3, the residual activity decreasing with increasing pH to give almost complete inactivation at *c.* pH 10.0. Pace (1930), using a partly purified preparation of trypsin, reports optimum stability at 50°C at pH 6.5. The difference in the results obtained by Kunitz and Northrop and those in Figure 2 may be due to the wide spacing of the experimental points on the curve presented by these authors or to the different conditions of inactivation. The results of Pace indicate that a protecting substance was present in the enzyme solutions since the greatest value obtained by Pace for the first-order reaction constant was of the order of 0.01 min^{-1} as compared with 0.7 min^{-1} in the present experiments (Fig. 8).

The inflexions in the curves of Figure 2 at pH 3.5, 6.0, 6.6, 6.8, 10.0, and 11.2 suggest that at least six different ionizable groups play a part in determining the thermal stability of trypsin. The peak of stability at pH 10.7 corresponds with the value obtained by Bier and Nord (1951) for the isoelectric point of the enzyme.

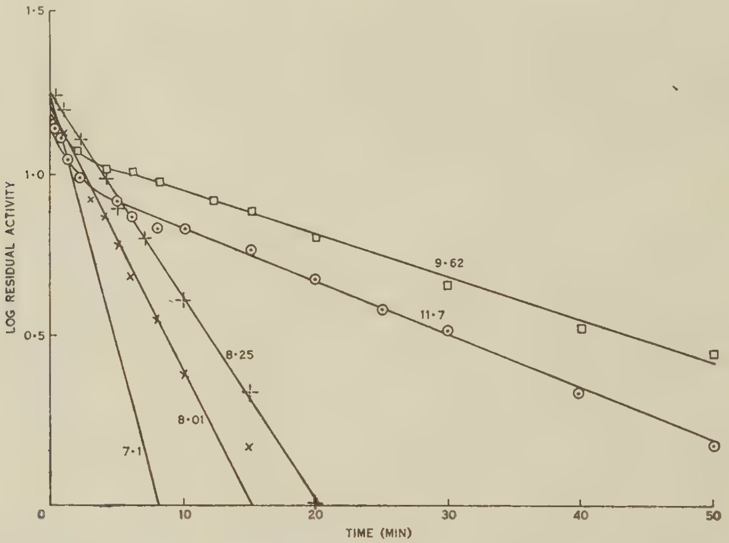

Fig. 9.—Kinetics of inactivation of trypsin at 40°C. pH values are indicated on the curves. Buffers were 0.1M borate (pH 7.0-9.0) and carbonate (pH > 9.0).

The apparent activation of trypsin by certain coordination complexes of Fe and its inactivation by others (Michaelis and Stern 1931), and the apparent activation of the enzyme by various cations observed by Kikuo Sugai (1944) and Bresler and Rosetzveig (1951), may be due in part to stabilization of the enzyme during the preliminary warming-up period and incubation with the substrate and in part to pH changes. The conclusions of the above authors and the assumption by Gorini (1951) and by Bier and Nord (1951) that divalent cations are necessary for the activity of trypsin are shown to be incorrect; the initial rate of hydrolysis of gelatin by the enzyme is unaffected by the presence or absence of cations such as Ca, Mg, or Mn in a substrate solution containing Na^+ as the only cation (Fig. 7). The stabilizing effects of a wide range of cations explain the greater yield of trypsin from trypsinogen in the presence of various cations (McDonald and Kunitz 1941).

In general the present results bear out those of Rona and Kleinmann (1928) except that the trivalent and tetravalent ions are effective stabilizing agents at even lower concentrations than these authors suggest. The greater efficacy of the cations of high valence may be due in part to the concentration of polyvalent ions at the protein-water interface as predicted by the Gibbs-Donnan theory (Danielli 1949). However, since the tetra- and trivalent cations all have pK_1 values considerably below the experimental pH values the ions would be present largely in the form $M(OH)^{++}$, $M(OH)^{+}_{2}$, and $M(OH)_3$ and the considerable difference between the concentrations of tri- and divalent cations required to provide a certain degree of stability cannot be adequately explained on this basis.

It seems more probable that the cations coordinate with certain groups on the protein. At the lowest concentration of divalent cations used in these experiments (1.5×10^{-4}M) the order of their stabilizing effects was Be > Cu > Zn > Cd > Co > Mg > Mn > Ca > Ba > Ni > Hg (Fig. 4). Except for Ni and Hg, which probably inhibit to some extent even at 1.5×10^{-4}M, this series resembles that for ease of formation of complexes with 8-hydroxyquinoline, acetylacetone, and salicylaldehyde (Maley and Mellor 1949*a*) and with amino acids (Maley and Mellor 1949*b*; Albert 1950; Monk 1951). On arranging these cations in order of their inactivating effects at 0.05M, namely, Hg > Ni > Cu > Be > Zn > Cd > Co > Mn > Mg > Ba > Ca, a comparable series is obtained. The two series can only approximate the true order of stabilization or inactivation owing to overlapping of the two effects, variations in the pH ranges at which the cations were tested, and precipitation of some of the cations as hydroxides in the more concentrated solutions.

Pauling (1948), on the basis of his neutrality theory, suggests that the ability of metals such as Fe, Cr, and Al to form trivalent ions is a result of their ability to form with the solvent coordinated complexes in which the bond between the metal and oxygen of the water is partly covalent. He considers that the non-coordinated form of the ions does not exist as such in solution. The formation of a coordinate complex between trypsin and a cation would therefore involve displacement of water from the complex. In complexes of ferric ions with other ligands such as tartrate or pyruvate the ligand would not be displaced as readily as water, and the ability of such a complex to stabilize the enzyme would depend on the relative affinities of the cation for the ligand and enzyme, and on their concentrations. The increase in stability of trypsin with decreasing concentrations of tartrate and pyruvate in solutions containing ferric ions held in solution by these complexing agents (Table 6) is in accord with this view.

Uncharged amino, guanidino, indole, or imidazole groups of the enzyme proteins would be expected to displace water from the cation complex more readily than phenolic, carboxyl, or alcohol groups (Fyfe 1952), and the increase in affinity of trypsin for cations with increasing pH conforms with this view. Thus the increase in affinity for cations over the pH range 6.0-7.5 (Fig. 2) could be due to the formation of metal complexes as protons are removed from the histidine side chains. Groups coordinated in this way could still form hydrogen bonds (Fyfe 1952) which may in fact be strengthened by the increased charge on the hydrogen atoms of the ligand (Pauling 1945). The reduction in the overall positive charge of the enzyme molecules with increasing pH would also facilitate the approach of the cation to the protein.

The manner in which stability of the enzyme is increased on formation of such a complex may be explained by two hypotheses. The first postulates that the cation, in forming a complex, increases the strength of certain labile hydrogen bonds either, as suggested above, by increasing the positive charge on amino or imino groups, or by providing additional cross linkages in the protein. The second hypothesis, based on the views of Levy and Benaglia (1950) concerning denaturation of ricin, suggests that each ionized form of the protein denatures at a characteristic rate and not necessarily via the same path. If the most stable arrangement of the molecule chelates preferentially with cations, this stable form will predominate at the expense of the unstable forms according to the Law of Mass Action, and the apparent pK values of the protein will be changed. In this way, when the pH of such a protein solution is raised, it may be possible for the molecule to pass from one pH zone of stability to another without assuming the intermediate unstable configuration to an appreciable degree. Stabilization according to both hypotheses could operate concurrently. The fall in pH of trypsin solutions in the pH range 7.0-9.5 on adding to the enzyme a solution of a salt at the same pH suggests that the second hypothesis is operative. It could be ascribed to the formation of a complex between the metal ion and the stable form of the protein having the primary amino groups or lysine amino groups in the uncharged condition, with a consequent dissociation of the ionized form of the protein during complex formation to yield protons. In the pH range 3.0-6.5, the presence of optimal concentrations of cations limits the formation of reversibly inactivated enzyme (Fig. 2) and this may also be a simple mass action effect.

Figures 8 and 9 show that between pH 7.0 and 8.0 the inactivation of trypsin is first-order with respect to trypsin, the maximum rate of inactivation being at *c.* 7.1. At pH values outside this range the plot of log (residual activity)-time is initially curvilinear but approaches a rectilinear relationship as inactivation proceeds. More than one type of equation can be fitted satisfactorily to such a curve but of these the most probable is that derived from a reversible inactivation, such as that demonstrated by Kunitz and Northrop (1934), together with an irreversible denaturation of the native protein thus:

$$\begin{array}{c} P \rightleftharpoons P', \\ \downarrow \\ P'' \end{array}$$

where P is the native enzyme, P′ the reversibly inactivated form, and P″ the irreversibly inactivated form. If k_1, k_2, and k_3 are the rate constants for the reactions

$$P \rightharpoonup P',$$
$$P' \rightharpoonup P,$$

and

$$P \rightarrow P'',$$

and $[P_0]$ is the initial concentration of the enzyme

$$\begin{aligned}\frac{d[P]}{dt} &= -(k_1+k_3)[P]+k_2[P'] \\ &= -(k_1+k_3)[P]+k_2([P_0]-[P]-[P'']) \\ &= -(k_1+k_2+k_3)[P]+k_2[P_0]-k_2k_3\int[P].dt.\end{aligned}$$

Integrating,

$$[P] = Ae^{-mt}+Be^{-nt}, \qquad \ldots \quad \ldots \quad \ldots \quad (1)$$

where A and B are constants such that $A + B = [P_0]$, and m and n have the form

$$\frac{k_1+k_2+k_3\pm\sqrt{[(k_1+k_2+k_3)^2-4k_2k_3]}}{2}.$$

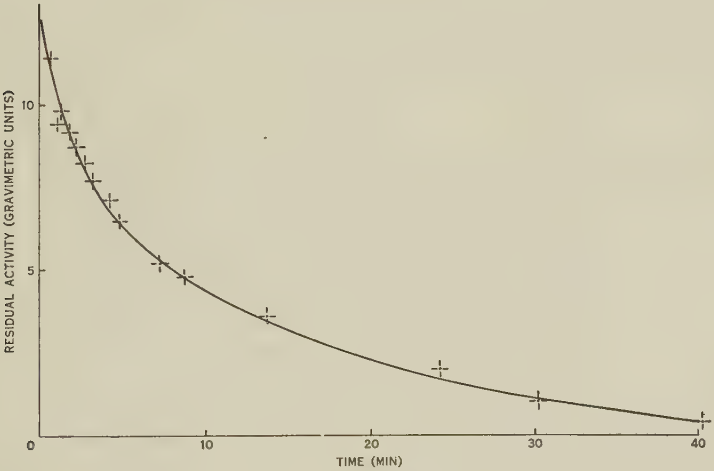

Fig. 10.—Comparison of inactivation of trypsin at pH 6.4 with the equation $[P] = 3.5e^{-0.711t} + 8.9e^{-0.068t}$, where [P] is the residual tryptic activity and t the time (min).

If the reversibly inactivated form of the enzyme were to change back to the native form on addition to the substrate the activity measured would be that of ([P] + [P′]).

Since

$$\begin{aligned}\frac{d([P]+[P'])}{dt} &= -\frac{d[P'']}{dt} = -k_3[P], \\ [P]+[P'] &= -k_3\int(Ae^{-mt}+Be^{-nt}) \,.\, dt \\ &= k_3(Am\, e^{-mt}+Bn\, e^{-nt}). \qquad \ldots \quad \ldots \quad (2)\end{aligned}$$

An equation having the form of (1) and (2) may be fitted satisfactorily to the kinetic data shown in Figures 8 and 9. Thus Figure 10 compares experimental values obtained at pH 6.4 and 40°C with an equation of this form.

In view of the first-order kinetics of the inactivation at pH 7.1, and the low concentration of trypsin used (10^{-6}M), of which less than 10 per cent. would be in the reversibly inactivated form at pH 7.1 (Kunitz and Northrop 1934) it is unlikely that irreversible inactivation of the enzyme is due to digestion of the reversibly inactivated enzyme by the active form. In the digestion of other protein substrates considerably higher concentrations of the substrate are required for the rate of hydrolysis to remain constant over a period of 10 min at 40°C. Furthermore, the maximum rate of irreversible denaturation takes place at a pH of *c.* 7.1 where the present experiments (Figs. 8, 9) and those of Kunitz and Northrop (1934) indicate the formation of reversibly inactive trypsin to be a minimum. Since the optimum pH for the hydrolytic action of trypsin is certainly higher than 7.1 maximum inactivation would be expected to occur at a higher pH. Furthermore, the initial rate of irreversible inactivation of trypsin at pH 7.1 is greater than the greatest initial rate of reversible inactivation under the conditions investigated in these experiments (Figs. 8, 9). This also suggests that the reversibly inactivated enzyme is not an intermediate in the formation of the irreversibly inactivated form.

TABLE 7

EFFECTS OF CATIONS ON THE FORMOL TITRATION

Titration of 5 ml enzyme-substrate mixture (50 ml 5 per cent. casein in collidine buffer at pH 7·6+2 ml trypsin solution) + 2 ml 0·05M cation solution + 3ml 36 per cent. formaldehyde, with 0·02N NaOH. The values given are differences between titres for an enzyme-substrate mixture held at 100°C during mixing and for a similar mixture incubated 15 min at 20°C before adding cations

Cation	Titration Difference (ml)
Nil	2·23
Ca	2·04
Mg	1·61
Ba	1·78

The differences in results of the present investigation and those obtained by Gorini (1951) and Bier and Nord (1951) can be explained by several differences in experimental conditions. Gorini's results are very similar to those obtained in the presence of sodium borate and sodium chloride (Table 3) and it is probable that his use of 0.1M sodium borate buffer masked the stabilizing effects of Mg^{++} and other cations. His use of trypsin at four times the concentration used in the present experiments would also tend to enhance the stabilizing effect of Ca^{++} and decrease that of Mg^{++} (Table 5). The differences in experimental conditions used by Bier and Nord and the manner in which they would affect the results are:

(1) A lower temperature and a longer time of heating were used by Bier and Nord and, although the concentration of trypsin used was not stated, experiment showed that they probably employed about 200 times the concentration of trypsin used in the present experiments. Table 6 shows that each of these factors would tend to increase the stabilizing effect of Ca^{++} and decrease that of Mg^{++}.

(2) The formol titration method was used to estimate residual protease activity. Investigation showed (Table 7) that the titration differences obtained with a trypsin-casein mixture before and after incubation differ according to the cations present. This effect would also tend to lessen the apparent stabilizing effects of Mg^{++} and Ba^{++} as compared with that of Ca^{++}.

(3) The concentration of cations used by Bier and Nord was higher than in the present experiments. At the concentration used (0.1M) many cations would be inhibitory (Fig. 4). Moreover, 0.05M borate or glycine buffers were used in most of the experiments by Bier and Nord, while in experiments with unbuffered solutions it was not stated whether the pH values quoted were those of the initial solutions or of the final mixture of enzyme and electrolyte.

Of these differences in technique the first appears to be the most important. The conditions used by Bier and Nord favoured autodigestion of the enzyme whether the substrate was the native enzyme, a reversibly inactivated form of the enzyme, or an irreversibly inactivated form. Possibly all three forms of the enzyme are digested to some extent. Cunningham *et al.* (1953) demonstrated the existence of a complex variation of the sedimentation rate of trypsin with enzyme concentration and pH. They suggested that in the pH range in which trypsin was active the protein existed in a concentration-dependent monomer-polymer equilibrium tending toward polymerization as protein concentration increased. In very dilute solutions, such as were used in the present study, the enzyme would be present largely as the monomer. The results suggest therefore that the monomer is thermally unstable, and its inactivation prevented or retarded by suitable concentrations of any cation. The dimer, on the other hand, is either readily hydrolysed by active molecules of the monomer, or constitutes an enzyme-substrate complex as suggested by Cunningham *et al.* (1953). Ca^{++} and Mn^{++} may decrease the formation of this complex.

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PURIFICATION AND PROPERTIES OF THYROID PROTEASE

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Summary

Thyroglobulin, purified to electrophoretic homogeneity by fractional precipitation of hog thyroid extracts with ammonium sulphate, has been shown to retain appreciable proteolytic activity.

By solvent-salt fractionation thyroid protease has been obtained substantially free from thyroglobulin.

The enzyme has optimum activity within the range pH 2.5-3.5. It is not activated by sulphydryl reagents, metal ions, trypsin, or thyrotrophic hormone.

I. INTRODUCTION

It seems now well established that the thyroid gland secretes L-thyroxine, as such, into the blood stream where it circulates in loose attachment to protein (Taurog and Chaikoff 1948; Leblond and Gross 1949; Taurog, Chaikoff, and Tong 1950; Rosenberg 1951; Gordon *et al.* 1952). Tri-iodothyronine is also present in normal plasma (Gross and Pitt-Rivers 1952).

There is, however, no certainty as to the structure of the thyroid hormone when it acts peripherally. Moreover, clarification is needed in regard to the mechanisms which effect the liberation of thyroxine from the more complex forms in which it is stored in the thyroid gland—although it might be assumed that simple proteolysis would be the prime motivator in this process. Gersh and Caspersson (1940), as a result of histochemical studies of thyroid glands of guinea pigs under different physiological conditions, formed the opinion that, in the normal animal, the production of colloid is a continuous process and suggested that a protease secreted by the gland cell may digest the colloid in the lumen to products—polypeptides or peptones—capable of rapid transfer across the cell membrane. In extending these observations, de Robertis (1941) was able to show that the colloid extracted from single thyroid follicles possessed appreciable proteolytic activity while Dziemian (1943) claimed that such activity varied with fluctuations in the physiological status of the animal.

In the course of experimental work with purified thyroglobulin we were able to confirm the existence of a close thyroglobulin-enzyme association by showing that such preparations still exhibited appreciable proteolytic activity even though they behaved on electrophoresis as homogeneous proteins. This led to attempts to separate the enzyme component(s) both from such purified thyroglobulin and from cruder material and to devise methods for its preparation and purification. The results of a study of the general properties of the protease are also reported here, while its application in an investigation of the enzymic breakdown of thyroglobulin labelled with ^{131}I will be reported in detail later.*

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II. Materials and Methods

All chemicals used were of A.R. grade and all solvents were purified by standard methods.

Purified Thyroglobulin.—This was prepared according to the procedure of Derrien, Michel, and Roche (1948) which involves fractional precipitation of a saline extract of thyroid glands with $(NH_4)_2SO_4$, the fraction soluble at 37 per cent. saturation and insoluble at 41 per cent. saturation being retained. Further treatment of the end-product so obtained was omitted in this work although in one instance (batch I) the complete fractionation procedure was followed through a second time. Fresh hog thyroids were used.

Table 1

COMPARISON OF THE PROTEOLYTIC ACTIVITY OF PURIFIED THYROGLOBULIN AND OF THYROID ENZYME PREPARATIONS (METHOD 1)

Preparation	Batch No.	Nitrogen (mg/ml test solution)	Iodine (μg/ml test solution)	$\frac{N}{I}$	Activity (m-equiv. × 10⁴ tyrosine liberated per mg N)
Thyroglobulin	H	3·70	139	27	8·9
Thyroglobulin	I*	3·44	—	—	6·3
Thyroglobulin	I†	4·47	134	33	5·7
Thyroglobulin	J	6·15	312	20	7·1
Thyroglobulin	L	3·00	105	29	6·0
Enzyme	2	0·25	2·2	114	57·6
Enzyme	6	0·28	1·5	187	63·6
Enzyme	7	0·26	1·1	236	55·4

* After first fractionation with $(NH_4)_2SO_4$.
† After second fractionation with $(NH_4)_2SO_4$.

Thyrotrophic Hormone.—Two specimens were used—one prepared by Schering A.G. and the other by Parke-Davis (preparation No. 099931) containing 34 and 9-10 Junkmann-Schoeller units/mg respectively.

Proteolytic Activity.—This was tested by the method of Anson (1938) with minor modifications—0.5 ml of enzyme solution was incubated with 2.5 ml of 2 per cent. haemoglobin as substrate for 30 min at 37°C; the supernatant, following precipitation with trichloracetic acid, was assayed with the phenol reagent of Folin-Dennis and results expressed as m-equiv. × 10^4 of tyrosine liberated. The haemoglobin was preserved as a freeze-dried powder.

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* A preliminary account of the results of this work was given at the 1951 Oxford Isotope Conference (Gordon *et al.* 1953).

pH.—Throughout the work pH was checked with the glass electrode. In testing the pH of alcoholic solutions the procedure of Cohn *et al.* (1950) was adopted and these were buffered by the addition of 2.5 ml/l of a stock solution of the required pH; the buffers used were acetate (4.3M) for pH 4.0 and mixtures of K_2HPO_4 (2M) and NaH_2PO_4 (4M) in the appropriate proportions to give pH 6.0 and pH 8.0 on dilution.

Iodine and Nitrogen Assays.—Iodine values were determined either by the semi-micro method of Astwood and Bissell (1944) or by the micro method of Barker (1950) and nitrogen by the micro-Kjeldahl procedure with selenium as catalyst.

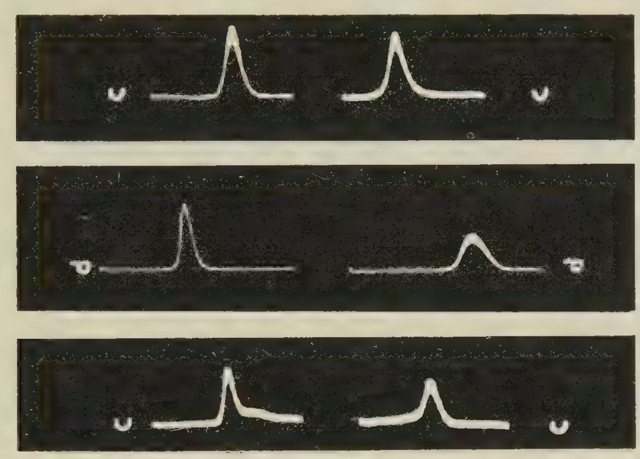


Fig. 1.—Electrophoretic tracings of thyroglobulin preparation I. Top, pH 5.70; middle, pH 7.65; bottom, pH 9.94. L.H.S.: ascending boundary. R.H.S.: descending boundary.

III. Experimental and Results

(*a*) *Proteolytic Activity of Thyroglobulin Preparations*

Results of determinations of the proteolytic activity of thyroglobulin preparations are listed in Table 1.

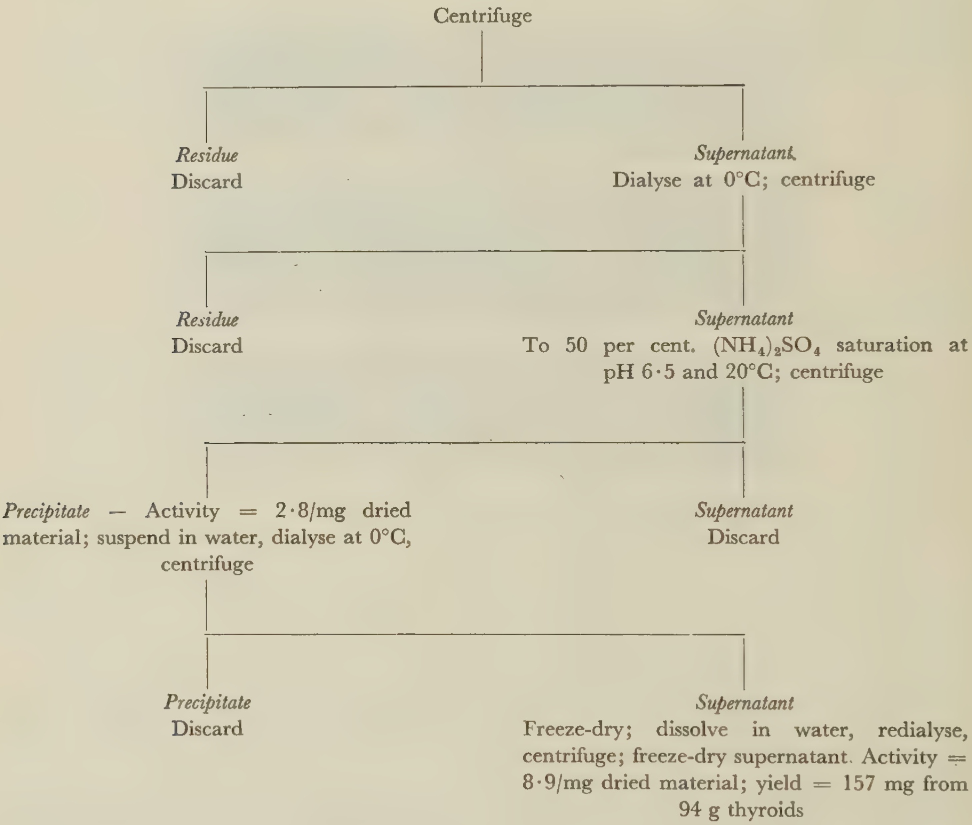
The proteolytic activities, expressed in arbitrary units,* are small but regular in value. Moreover, there was no significant change in content when one of the samples, batch I, was refractionated with $(NH_4)_2SO_4$. In accord with the observations of Derrien *et al.* (1949) on specimens of thyroglobulin similarly prepared, this material was found to be electrophoretically homogeneous. The sample was run at pH levels of 5.70, 7.65, and 9.94 (Fig. 1). No evidence of more than one component was obtained; an apparent second component in tracing *c*, pH 9.94, is a boundary anomaly usual with this buffer (Michaelis universal veronal buffer) and which migrates slowly at this pH.

* One unit is the amount of enzyme required to liberate 10^{-4} m-equiv. tyrosine in 30 min at 37°C.

(*b*) *Preparation of Protease*

Preliminary studies indicated that preferential denaturation of the thyroglobulin would facilitate the separation of the protease component. Consequently it was decided to use cruder material, prepared by precipitation of thyroid extracts with $(NH_4)_2SO_4$, rather than purified thyroglobulin, as the starting point for fractionation studies.

Method 1.—Hog thyroids; extract with 4 vol. 60 per cent. glycerol in Waring Blendor. Add toluol. Overnight at 0°C. Activity of extract = 0.112/mg thyroid.



Centrifuge

Residue
Discard

Supernatant
Dialyse at 0°C; centrifuge

Residue
Discard

Supernatant
To 50 per cent. $(NH_4)_2SO_4$ saturation at pH 6·5 and 20°C; centrifuge

Precipitate — Activity = 2·8/mg dried material; suspend in water, dialyse at 0°C, centrifuge

Supernatant
Discard

Precipitate
Discard

Supernatant
Freeze-dry; dissolve in water, redialyse, centrifuge; freeze-dry supernatant. Activity = 8·9/mg dried material; yield = 157 mg from 94 g thyroids

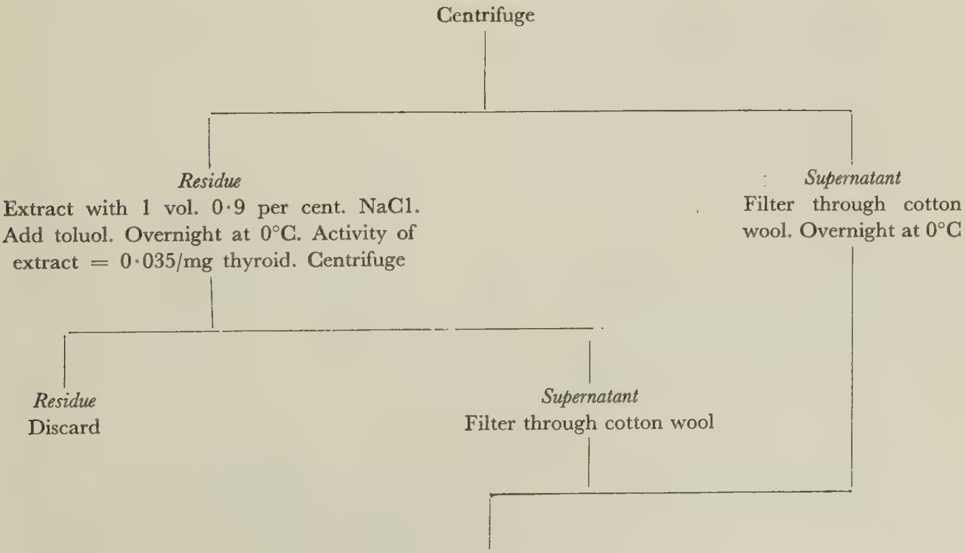
The activities of three batches of enzymes prepared by this method are compared with those of four batches of thyroglobulin in Table 1. However, this method proved unreliable as it was partly dependent on separation of insoluble material—chiefly denatured thyroglobulin—during dialysis and this precipitation was not always complete.

As extracts prepared with glycerol, phosphate buffer at pH 7, and isotonic sodium chloride were found to be of equal activity, saline was used in all future extractions. The crude material for fractionation was then obtained by precipitation at pH 6.5 with $(NH_4)_2SO_4$ at 45 per cent. saturation at which concentration removal of the enzyme from solution was complete.

Method 2 (*Solvent-Salt Fractionation*).—In a preliminary experiment in which the early stages of the plasma-fractionation procedure of Cohn *et al.* (1950) were carried out, the proteolytic activity was found in the precipitate from a 0.9 per cent. NaCl solution at 20 per cent. ethanol concentration which contained the bulk of the protein. More extensive studies along these lines with methanol also failed to effect a separation when pH levels of 4, 6, 7, or 8 and saline concentrations of 0.01 or 0.1 per cent. were used.

However, when a comparison was made of the solubility of the crude protein in distilled water and in isotonic saline, it was found that, on the addition of dilute acid to a solution in distilled water, precipitation occurred at the isoelectric point of thyroglobulin—pH 4.5—and, as the pH was lowered, the precipitate redissolved. On repeating this procedure using a solution of the crude protein in isotonic saline, precipitation occurred at pH 4.5 but the precipitate appeared to be completely insoluble even at pH 3.2. However, the clear supernatants obtained by centrifuging such solutions below pH 4.5 were found to possess appreciable proteolytic activity. When methanol was added to a solution of the crude protein in 0.1 per cent. NaCl to a concentration of 60 per cent., the precipitate suspended in 1 per cent. NaCl, and the pH adjusted to 3.5, about 60 per cent. of the total activity of the resulting suspension was found to be contained in the water-clear supernatant. The methanol appeared to effect a more rapid and complete denaturation of the bulk protein. These findings were utilized in the method of preparation finally adopted.

Procedure.—Extract glands with 3 vol. 0.9 per cent. NaCl in Waring Blendor. Add toluol. Overnight at 0°C. Activity of extract = 0.200/mg wet thyroid tissue.



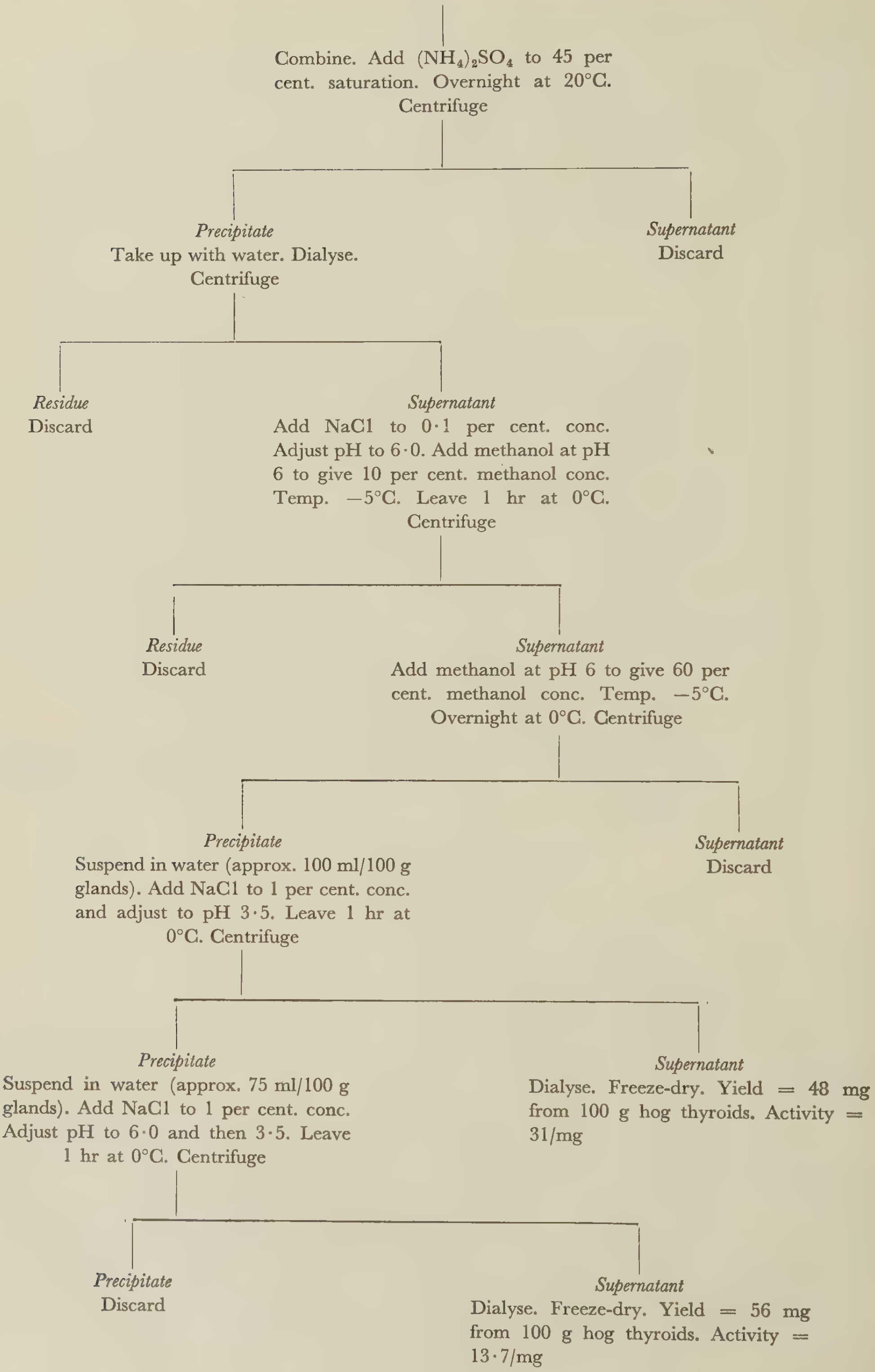
Centrifuge

Residue
Extract with 1 vol. 0·9 per cent. NaCl. Add toluol. Overnight at 0°C. Activity of extract = 0·035/mg thyroid. Centrifuge

Supernatant
Filter through cotton wool. Overnight at 0°C

Residue
Discard

Supernatant
Filter through cotton wool



Combine. Add $(NH_4)_2SO_4$ to 45 per cent. saturation. Overnight at 20°C. Centrifuge

Precipitate
Take up with water. Dialyse. Centrifuge

Supernatant
Discard

Residue
Discard

Supernatant
Add NaCl to 0·1 per cent. conc. Adjust pH to 6·0. Add methanol at pH 6 to give 10 per cent. methanol conc. Temp. −5°C. Leave 1 hr at 0°C. Centrifuge

Residue
Discard

Supernatant
Add methanol at pH 6 to give 60 per cent. methanol conc. Temp. −5°C. Overnight at 0°C. Centrifuge

Precipitate
Suspend in water (approx. 100 ml/100 g glands). Add NaCl to 1 per cent. conc. and adjust to pH 3·5. Leave 1 hr at 0°C. Centrifuge

Supernatant
Discard

Precipitate
Suspend in water (approx. 75 ml/100 g glands). Add NaCl to 1 per cent. conc. Adjust pH to 6·0 and then 3·5. Leave 1 hr at 0°C. Centrifuge

Supernatant
Dialyse. Freeze-dry. Yield = 48 mg from 100 g hog thyroids. Activity = 31/mg

Precipitate
Discard

Supernatant
Dialyse. Freeze-dry. Yield = 56 mg from 100 g hog thyroids. Activity = 13·7/mg

Certain preparations gave lower recoveries of purified enzyme. This is probably linked with the low protein concentrations during dialysis at the later stage of the preparation. In two of these preparations in which only small amounts of protease were recovered, the enzyme was of relatively high activity —90/mg—perhaps owing to removal of a contaminant by adsorption on the Cellophane.

The proteolytic activities and iodine contents of four specimens of protease prepared according to the above method are listed in Table 2. The fact that iodine is still present is presumably due to contamination and not to inherent association of iodine and the enzyme protein, while the variation in iodine content of the specimens probably reflects the nature of the thyroglobulin contaminant; thus in a parallel investigation the iodine values of a series of purified thyroglobulin samples were found to range between 0.15 and 0.86 per cent.

TABLE 2

PROTEOLYTIC ACTIVITY OF THYROID ENZYME PREPARATIONS (METHOD 2)

Preparation No.	Iodine (g/100 g)	Activity (m-equiv. × 10^4 tyrosine liberated per mg enzyme)
14	0·016	33·6
16	0·034	23·2
17	0·018	39·5
18	0·042	37·0

(*c*) *Properties of Protease*

The enzyme preparations made according to either method were white powders, readily soluble in water. Solutions were found to be without activity following heating at 100°C for 5 min at pH 3.5.

pH activity curves were determined for a number of specimens of protease, mostly within the pH range 2-4. Where tests were carried out at pH values above 4 it was found that the addition of urea to denature the haemoglobin substrate (as recommended by Anson 1938) inactivated the enzyme. Consequently, the following general procedure was used for the preparation of denatured substrate: haemoglobin powder was dissolved in water, the pH lowered to 1.15, and the solution left 1 hr at room temperature. Aliquots were then taken, adjusted to the required pH with 1N NaOH, and diluted to give a 2 per cent. solution. Owing to the strong buffering action of haemoglobin, individual adjustment to the correct pH was preferred to the addition of buffer solutions.

The results obtained with two specimens of enzyme prepared according to method 2 are shown in Figure 2. Although our earlier observations with cruder preparations had indicated a pH maximum at 3.5 in accord with Dziemian

(1943), present findings show a practically constant value between pH 2.5 and 3.5. In other tests it was found that the activity continued to drop from pH 4 to 6.

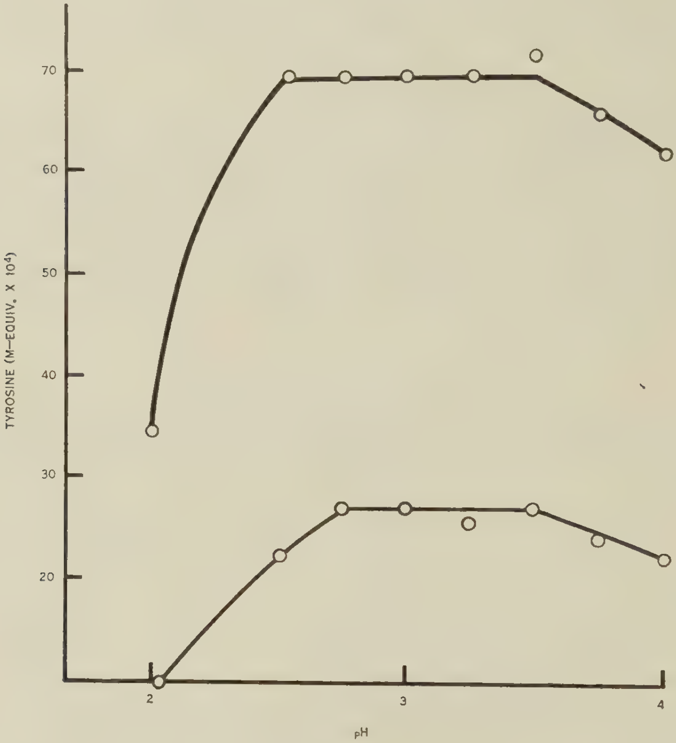


Fig. 2.—pH activity curves of purified protease, preparations 34 and 35 (method 2). Activity at pH 3.5 = 71.5 and 27.0 respectively.

The action of the protease was tested only on the substrates haemoglobin and thyroglobulin. On the latter protein, under the conditions of assay as applied to haemoglobin, the enzyme did not appear to exert any effect. However, that there is a well-marked action on this substrate was evident in the more intensive studies with longer periods of incubation in which thyroglobulin labelled with ^{131}I was utilized (unpublished data).

(*d*) *Experiments with Activators*

(i) Using crude thyroid extracts, Dziemian (1943) and Kamner, Peranio, and Bruger (1950) reported increased proteolytic activity in the presence of cysteine. The effect of incubation of the purified enzyme with cysteine and thioglycollic acid was therefore investigated. Results are listed in Table 3. In this table are also recorded the results of attempts to activate the protease by incubation with Mn^{++} (as $MnSO_4$). This metal was chosen in view of its recognized role as a cathepsin activator (Lehninger 1950) and particularly in consequence of the report by Deysach and Ray (1949) that Mn is preferentially absorbed by the thyroid gland. Enzyme solution plus activator was usually incubated for 2 hr and the activity subsequently compared with that of a control solution incubated at the same pH for a similar period.

TABLE 3

ACTIVITY OF ENZYME FOLLOWING INCUBATION FOR 2 HR WITH THIOGLYCOLLIC ACID, CYSTEINE, Mn^{++}, AND Ca^{++}

Preparations 8, 10, 12*a*, and 12*b* were obtained according to method 1, preparation 18*a* according to method 2

Preparation No.	Agent Under Test	Concentration of Agent	pH of Incubation	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme*
Crude NaCl extract	Thioglycollic acid	0·003M	3·2	7·8†
Crude NaCl extract	Control	—	3·2	9·3†
8	Thioglycollic acid	0·015M	3·85	7·1
8	Control	—	3·85	7·1
10	Cysteine	0·004M	3·5	15·5
10	Cysteine	0·008M	3·5	15·8
10	Control	—	3·5	15·4
12*a*	Cysteine	0·017M	3·7	19·5
12*a*	Control	—	3·7	17·9
8	Mn^{++}	0·02M	3·5	6·2
8	Control	—	3·5	7·2
8	Mn^{++}	0·02M	7·0	7·5
8	Control	—	7·0	7·2
8	Mn^{++}	0·02M	7·5	6·2
8	Control	—	7·5	6·1
12*b*	Mn^{++}	0·02M	6·95	11·2
12*b*	Control	—	6·95	11·2
12*b*	Mn^{++}	0·02M	3·5	8·3
12*b*	Control	—	3·5	11·2
18*a*	Ca^{++}	0·02M	‡	50·2
18*a*	Control	—	‡	50·2

* pH of test, 3·5. † Figures represent activity per ml extract. ‡ Not incubated.

In order to reduce to a minimum the high value for the blank determination usually obtained in experiments with cysteine, due to the reaction of this substance with the phenol reagent, the enzyme powder was either dissolved directly in one drop of cysteine hydrochloride solution of appropriate concentration (experiments with enzyme preparation No. 10) or in one drop of water and an equal volume of cysteine hydrochloride added (experiments with enzyme preparation No. 12*a*). At the conclusion of the incubation period, the solution of enzyme was diluted to the requisite volume before assaying with haemoglobin substrate.

It is apparent that, under these test conditions, no activation of the protease by any of these agents was observed. Results obtained with purified enzyme, therefore, do not confirm the claims of previous workers cited above using crude thyroid extracts. It should be noted, however, that Dziemian (1943) used a different protein substrate, edestin, in his assays. Incubation with Mn^{++} at pH 3.5 decreased somewhat the activity of the enzyme, although this effect was not seen when prior incubation was carried out at pH levels between 6.95 and 7.5. It may also be noted that we were unable to confirm the observation of Kamner, Peranio, and Bruger (1950) that proteolytic activity was increased in the presence of acetate (Watson and Trikojus, unpublished data).

TABLE 4

ACTIVITY OF ENZYME (PREPARATIONS 6*a*, 6*b*; METHOD I) FOLLOWING INCUBATION FOR 2 HR WITH THYROTROPHIC HORMONE (34 J.S. UNITS/MG)

	TSH Concentration (J.S. units/mg enzyme)	pH of Incubation	pH of Test	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme
Enzyme (6*a*)+TSH	4	3·6	3·5	13·0
Enzyme (6*a*)+TSH	4	3·6*	3·5	14·0
Enzyme (6*a*) control	—	3·6	3·5	14·1
TSH control	4	3·6	3·5	—
Enzyme (6*b*)+TSH	4	7·0	3·5	9·5
Enzyme (6*b*)+TSH	4	7·0*	3·5	10·9
Enzyme (6*b*) control	—	7·0	3·5	10·2
TSH control	4	7·0	3·5	—
Enzyme (6*b*)+TSH	4	7·0	7·0	—
Enzyme (6*b*)+TSH	4	7·0*	7·0	—
Enzyme (6*b*) control	—	7·0	7·0	—
TSH control	4	7·0	7·0	—

* Enzyme and TSH were incubated separately and combined for testing.

(ii) In view of the report (Dziemian 1943) that the proteolytic enzyme content of the thyroid gland is increased after administration of thyrotrophic hormone (TSH) to rats and guinea pigs (Watson and Trikojus, unpublished data 1951), it was decided to investigate the effect of TSH on the protease *in vitro* since TSH is known to exert its action within the thyroid gland. Enzyme + TSH were incubated together for 2 hr at two different pH levels and the activity of the solution subsequently compared with controls consisting of enzyme incubated alone and of enzyme and TSH incubated separately and combined for testing. TSH was also tested for proteolytic activity. Results are given in

Table 4. It can be seen that TSH neither possesses proteolytic activity nor does it influence the activity of the enzyme.

In order to determine whether there might be an accessory factor in thyroid tissue essential for activation of the protease by TSH, a cat thyroid was finely sliced and the slices held in Ringer solution. After removal of excess moisture, one aliquot of 50 mg was added to 4 ml of enzyme solution (preparation No. 7; 2 mg/ml) and an equal amount to 4 ml enzyme solution + 8 mg TSH (preparation No. 099931) in Warburg flasks which were then shaken for 4 hr at 37°C, the pH being 7.4. At the conclusion of the incubation period, the contents were centrifuged and 0.5 ml supernatant tested. The values obtained, 9.3 and 9.7 units/mg enzyme for presence and absence of TSH respectively, indicate again no effect of the hormone.

(iii) The influence of incubation together with another proteolytic enzyme, trypsin, on the activity of the thyroid protease was determined. Thyroid protease, dissolved in a solution of trypsin in 0.02M $CaCl_2$, was incubated at 37°C at pH 6.5 or 8.15. Aliquots were withdrawn at intervals and proteolytic activity tested at pH 3.5. Results are set out in Table 5.

TABLE 5

ACTIVITY OF THYROID PROTEASE (PREPARATION NO. 19; METHOD 2) FOLLOWING INCUBATION WITH TRYPSIN

Concentration of Trypsin (mg/ml)	pH of Incubation	Period of Incubation (min)	M-equiv. × 10^4 Tyrosine Liberated per mg Enzyme*
0·01	6·5	0	53·0
0·01	6·5	15	48·0
0·01	6·5	30	49·5
0·01	6·5	120	51·0
0·05	6·5	0	57·0
0·05	6·5	15	50·0
0·05	6·5	30	50·7
0·05	6·5	60	55·8
0·05	6·5	120	52·4
0·06	8·15	0	56·4
0·06	8·15	30	58·7
0·06	8·15	120	58·7

* pH of test, 3·5.

It is apparent that incubation with trypsin did not significantly influence the activity of the thyroid enzyme.

IV. DISCUSSION

The studies reported here provide indirect evidence that there is a close association of thyroglobulin with protease in the thyroid gland; they also indicate a procedure whereby the components can be separated. In unpublished experiments during which thyroid extracts were fractionated with ammonium sulphate, the proteolytic activity of each fraction was determined using haemoglobin at pH 3.5; whereas about half the total activity was found in the fraction insoluble at 42 per cent. and soluble at 37 per cent. ammonium sulphate saturation (i.e. purified thyroglobulin), the remainder was distributed between the fraction soluble at 42 per cent. and that soluble at 37 per cent. saturation. It is therefore possible that the proteolytic activity of thyroid tissue is not confined to a single protease although no attempt was made to characterize the other fractions.

Compared with the proteolytic enzymes of the digestive tract (Northrop, Kunitz, and Herriott 1948) the activities of even the purest thyroid protease preparations are relatively low; crystalline pepsin and trypsin would be respectively roughly 100 and 50 times as potent. However, our usual preparations appear to be about 40 times as active as a specimen of cathepsin isolated from thymus tissue by Maver and Greco (1949). A close comparison with the cathepsins of spleen as studied by Fruton and colleagues (e.g. Tallan, Jones, and Fruton 1952) was not possible owing to the different assay techniques used by these workers. It is of interest that Adams and Smith (1951) found that their most active preparation of proteinase I (pH optimum 3.8) isolated from pituitary extracts exhibited a proteolytic activity one-sixtieth that of crystalline trypsin when assayed against haemoglobin.

Activity studies with purified enzyme failed to demonstrate a pH optimum at 3.5 as reported by previous workers (e.g. Dziemian 1943) using crude extracts of the gland; moreover earlier claims of activation by sulphydryl reagents could not be confirmed. Negative results were also obtained with Mn^{++} and Ca^{++}. However, these findings are similar to those reported for the pituitary proteinases by Adams and Smith (1951) since cysteine, ascorbic acid, and various metal ions, including Mn^{++}, were found to be without effect on the activity of these enzymes. Of further interest is the failure to influence the activity of the thyroid enzyme by incubation with trypsin or with purified thyrotrophic hormone even in the presence of surviving slices of thyroid tissue.

V. Acknowledgments

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OSMOTIC PRESSURE AND DIFFUSION CONSTANT OF PROTEIN EXTRACTED FROM WOOL WITH UREA-BISULPHITE SOLUTIONS

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Summary

Wool treated with 8M urea, 0.4M $NaHSO_3$ at 60°C forms a polydisperse soluble protein of number-average molecular weight 12,000-16,000 as deduced from osmotic pressure measurements. The values lie within this range whether 20 or 70 per cent. of the wool is dissolved. The pH of measurement or of extraction has no effect on the molecular weight of the solute over the range 5.6-8.0. The diffusion constant, $D_{20,w}$, of the soluble protein is 4.5×10^{-7} $cm^2 sec^{-1}$.

I. Introduction

Solutions containing sodium bisulphite and high concentrations of urea have been widely used to render keratin partly soluble in an aqueous medium (Jones and Mecham 1943). The bisulphite causes fission of the disulphide bond of cystine with the formation of sulphydryl and S-cysteine sulphonate groups (Middlebrook and Phillips 1942) and the urea breaks internal hydrogen bonds in the protein.

Several workers (Mercer and Olofsson 1951; Olofsson 1951; Woods 1952; Ward 1952) have published determinations of molecular constants of wool dispersed in this solution but the results do not agree. By sedimentation and diffusion measurements Mercer and Olofsson found molecular weights for the wool molecule of 42,000 and 84,000 at pH 7 and 8 respectively, and axial ratios of 65:1 and 90:1, whereas Ward found, again from sedimentation and diffusion measurements, a molecular weight of 12,000-17,000 and an axial ratio of 45:1. From viscosity measurements Woods found that the molecule had an axial ratio of 10-16:1 and the molecular weight determined osmometrically was 30,000.

The present paper describes a study of the osmotic pressures and diffusion constants of wool extracts in urea-bisulphite solutions over the concentration range 0.5-3.5 per cent. of protein.

II. Experimental

(*a*) *Preparation of Wool Extracts*

The wool extracts were prepared by the following procedures:

(i) Solvent-scoured 64's Merino wool (50 g) was immersed at 60°C for 48 hr in 8M urea (300 ml) containing 0.4M $NaHSO_3$ (Merck Reagent Grade).

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The protein solution was then separated by centrifugation, the residue was extracted with a further 200 ml of urea-bisulphite solution, and the extracts were combined. During extraction the pH of the solution rose from the initial value of 5.6 to 7.8. About 15-20 per cent. of the wool was dissolved under these conditions. The extract was dialysed for 2 days against 2 l. of urea-bisulphite solution (8M urea, 0.4M $NaHSO_3$, 0.1N CH_3COONa) and then centrifuged at 25,000g for 1 hr to remove a small amount of aggregated material. By this means an optically clear solution was obtained. The pH of this solution was maintained above 5, as otherwise the solution became turbid, indicating gross aggregation of the protein.

(ii) Merino wool (64's, 6 g) was immersed for 8 hr at 60°C in 100 ml of 8M urea, 0.4M bisulphite solution. The mixture was homogenized in a Waring Blendor for 20 min and the extraction continued at 60°C for a further 16-24 hr. With this method more than 60 per cent. of the wool dissolved; with repeated extraction and homogenization at least 85 per cent. could be dissolved, as determined by estimation of the protein in solution. The increase in solubility was almost certainly due solely to the mechanical disintegration of the swollen fibre whereby the solubilizing agents could penetrate the cortex much more readily. It is interesting in this connection that wool ground in a Wiley mill prior to extraction was not significantly more soluble in the reagent than the intact fibre.

For diffusion measurements 20 ml of the stock solution of protein, prepared by the first of the above methods, was dialysed against 2 l. of urea-bisulphite solution containing 0.1M CH_3COONa, 0.1M CH_3COOH, and 0.05M NaCl. More dilute solutions were obtained by dilution of the stock. Osmotic pressure measurements were carried out using similar solutions or solutions prepared by the second method. pH's were all measured using a glass electrode assembly and refer to values obtained in 8M urea.

(*b*) *Estimation of Protein Concentration*

The concentration of protein was determined by dialysing 5-ml samples against running water and weighing the precipitated protein after drying at 105°C for 18 hr. Attempts were made to use the modified Folin-Wu colorimetric method (Lowry *et al.* 1951) since a similar method was found satisfactory by Burk and Greenberg (1930) for various proteins dissolved in urea solutions, but the presence of sodium bisulphite caused irregular colour formation. Destruction of the bisulphite by boiling with acid, by oxidation with bromine water followed by boiling, or by oxidation with hydrogen peroxide followed by destruction of excess peroxide with a minute amount of catalase did not satisfactorily overcome this difficulty.

(*c*) *Measurement of Viscosity*

Viscosities were measured in Ostwald viscometers (British Standard Nos. 0 and 1) and densities in a Sprengel pyknometer.

(*d*) *Measurement of Osmotic Pressure*

The osmotic pressure was measured in the osmometers of Adair (1925) and Bull (1941) and in a modified Hepp osmometer designed by Scatchard *et al.* (unpublished data). All measurements were made in a constant-temperature bath at 27.2°C. "Cellophane" tubing (Visking 18/32 in.) identical with that used for dialysis of the protein solution was used in the Adair and Bull osmometers, and a period of 1 wk was usually required for equilibrium to be established. In the Hepp osmometer "Cellophane 300 PT" was found more satisfactory than other membranes such as "Cellophane 600 PT" and collodion. Measurements by both dynamic and static methods were completed within 3-4 hr.

(*e*) *Measurement of Diffusion Constant*

Diffusion constants were measured in a standard LKB 3021 Tiselius apparatus equipped with a diagonal-slit Schlieren system. The 10-cm intermediate cell sections were used. The boundaries were moved mechanically to the centre of the cell and then sharpened according to the technique of Kahn and Polson (1947). Because of the high viscosity of the solutions, the sharpening was not as successful as it might have been, but the "zero time" of the experiments was reduced considerably from that found without sharpening the boundaries. Diffusion was allowed to proceed for *c.* 7 days at 7.5°C. At the conclusion of each experiment tracings of enlarged images of the boundaries were made on squared paper for measurement.

III. RESULTS

(*a*) *Osmotic Pressure Measurements*

The molecular weights were calculated from the extrapolated value of P/C (P = osmotic pressure, C = concentration of protein) using the formula

$$M = RT/(P/C)_0,$$

where M is molecular weight, R the gas constant, and T the absolute temperature. If P is in cm H_2O and C in g/l the expression becomes, at 27.2°C,

$$M = 25{,}475/(P/C)_0.$$

The range of protein concentration covered was 4-20 g/l, the lower limit being set by the sensitivity of the analytical method used. The values of P/C obtained with the Adair and Bull type osmometers showed a considerable spread (Table 1) and led to a mean P/C of 2.06 ± 0.22 (S.D.) with no certain concentration dependence. The corresponding molecular weight is 12,400. On the other hand the values obtained with the Hepp osmometer showed very little spread, no concentration dependence, and a $(P/C)_0$ value of 1.57 ± 0.04 (S.D.), which gives a molecular weight of 16,200. It was noticed with the Adair and Bull type osmometers that after some days, when apparent equilibrium had been reached, there was often a slight fall in pressure. With the Hepp osmometer there was a slow fall of osmotic pressure with time but this did not affect the results as measurements were usually completed in 3-4 hr. Scatchard (1952) reported similar effects using a Hepp osmometer with isinglass and stated that extrapolation to zero time gave accurate values for molecular weights. A similar procedure was found to be valid in the present instance. The slight decrease in osmotic pressure is attributed to a slow diffusion of protein through the membrane. Some of the molecules of such polydisperse systems certainly lie in the critical range for diffusion through the "Cellophane."

TABLE 1

OSMOTIC PRESSURE MEASUREMENTS

In experiments 1-4 about 20 per cent. of the wool was dissolved but in experiments 5 and 6 the amount was 70 per cent.

	Experiment No.	Concentration (g/l)	pH	P/C
Adair and Bull osmometers	1	11·4	5·4	2·22
		11·5		2·17
		8·0		2·43
		7·9		2·38
		4·2		1·89
	2	14·66	5·6	1·91
		14·56		1·92 Mean 2·06±0·22*
		7·90		1·83 M = 12,400
		7·78		1·82
		4·74		1·84
	3	7·80	5·7	2·00
		3·90		2·32
		3·90		2·08
Hepp osmometer	4	8·86	5·6	1·57
		8·86		1·60
		8·86		1·58
	5	18·1	5·6	1·55
		9·05		1·49 Mean 1·57±0·04*
		4·53		1·56 M = 16,200
	6†	18·8	5·8	1·57
		17·5	7·9	1·61

*Standard deviations.

† The initial pH of the urea-bisulphite was 5·8 but during extraction of the wool the pH rose to 7·8. The two measurements were carried out on samples dialysed against buffers of pH 5·8 and 7·9 respectively.

(*b*) *Diffusion Measurements*

The shortcomings of the Tiselius apparatus for measuring diffusion constants are well known. They arise chiefly from the facts that the cylindrical lens is uncorrected and that the boundary has to be moved after its formation. In the present work the use of a boundary-sharpening device partly compensated for the second difficulty. However, the maxima of the diffusion curves were not particularly well defined, especially near the beginning of an experiment.

Diffusion constants were usually calculated from both limbs of the apparatus by both the height-area (D_h) and second moment (D_m) methods. In Table 2 the two values from each experiment have been averaged. All values were corrected to diffusion in water at 20°C. For calculating D_h a mean area was used. It will be seen that there is no significant concentration dependence in the values of D_h. The values for both D_m and D_h were derived from the gradient of the root mean square line through about six points over the interval 0-10,000 min. The points for D_h fitted these closely but there was a tendency for D_m to diminish somewhat in the later stages of the diffusion process ($t > 5000$ min). This effect has been observed previously (Neurath 1942); in the present investigation at least it may be due to slow aggregation of the protein. However, all points were given equal weight in the calculation of D_m, which no doubt contributed largely to the wide variation encountered in the results.

Table 2

DIFFUSION CONSTANTS OF WOOL PROTEIN

Experiment No.	Concentration (g/l)	$D_m \times 10^7$	$D_h \times 10^7$
7	17	4·39	4·38
7*a*	35	4·98	4·65
7*b*	9	4·09	4·50
7*c*	9	—	4·52
		Mean 4·49	Mean 4·51

IV. Discussion

The molecular weights 12,000 and 16,000 derived from osmotic pressure measurements appear to agree with the value of 14,000 reported by Ward (1952). However, Ward employed values of sedimentation (S) and diffusion constants (D) determined at 3 per cent. concentration in his calculations. The validity of this procedure depends upon S and D having similar concentration dependence. In the present investigation it was shown that D_h varied little with concentration whereas Ward found a strong concentration dependence of S upon C. When this is taken into account it appears that Ward's molecular weights should be approximately doubled. This would be much more reasonable, since if Ward's weight-average value and our number-average value were approximately the same the protein solution would be fairly homogeneous in molecular size, which is most unlikely.

The values reported in the present paper are also lower than the approximate value of 30,000 reported by Woods (1952), but this would be accounted for by his use of a more porous membrane, since he found that during the attainment of equilibrium some 30 per cent. of the protein passed through the collodion. Ward found that 10 per cent. of his wool extract was dialysable through "Cellophane." In the present study the amount of protein passing through "Cellophane" was too small to be detected with protein precipitants.

The value 12,000-16,000 is much lower than Mercer and Olofsson's value of 84,000 obtained at pH 8 and Olofsson's value of 42,000 at pH 7. Olofsson (1951) stated that this high-molecular-weight fraction was limited to 10 per cent. or less of the total weight of wool. If this were so, it would not appreciably affect the number-average molecular weight. Mercer and Olofsson carried out their sedimentation and diffusion measurements in 25 per cent. urea (approximately 4M), although they had used 8M urea for the extraction. When the urea concentration of our extracts was reduced below *c.* 6M a considerable increase in the turbidity of the solution invariably occurred, suggesting that the protein had aggregated. This conclusion is supported by the low value reported by Mercer and Olofsson for the diffusion constant ($D_h = 1.61 \times 10^{-7}$) compared with the present value of 4.5×10^{-7}.

It is further shown that there was no difference in molecular weight of the protein extracted at pH 5.6 and at pH 8.0, which is contrary to Olofsson's results.

Osmotic pressure measurements gave the same molecular weight whether 20 or 70 per cent. of the wool protein dissolved and thus revealed no obvious fractionation of the protein in the extraction process. The mean diffusion constant, 4.5×10^{-7} cm^2 sec^{-1}, is only slightly lower than Ward's value of 4.9×10^{-7}. Because of the high degree of polydispersity of the wool protein solution, axial ratios calculated from molecular weights and diffusion constants using Perrin's equation are of doubtful significance. Various authors have applied such treatments and obtained results indicating extremely asymmetric molecules. Recent theoretical developments (Fessler and Ogston 1951; Scheraga and Mandelkern 1953)* make it probable that many axial ratios calculated in the literature are too large, and more exact data on actual hydration of well-defined proteins or protein fractions are necessary before the question of axial ratios of protein molecules in solution can be finally settled. Applying the theory of Scheraga and Mandelkern to these urea-bisulphite extracts of wool, and using the viscosity results of Woods together with the diffusion constant and corrected sedimentation molecular weight of Ward (approx. 28,000), it was found that values of the parameter β are obtained which correspond to molecules of low asymmetry.

Reduction to normal coordinates of several diffusion curves from experiment 7 showed very little deviation from a standard Gaussian curve. Experiment 7*a*, however, showed rather more. No definite conclusions can therefore be reached concerning the homogeneity of the protein.

The small difference in average D_m and D_h values in Table 2 does not indicate that the extracted wool protein is monodisperse. Gralén (1941, 1947) has shown that a solute must be grossly polydisperse to make D_m and D_h differ by more than 10 per cent., and the individual values of D_m in Table 2 vary so much that no great reliance can be placed upon the average.

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* We are indebted to Mr. E. F. Woods for drawing our attention to these papers.

Experiments by several workers (Olofsson and Gralén 1947; Mercer and Olofsson 1951; Ward 1952) as well as some unpublished experiments from this laboratory showed that treatment of wool at high pH (>12) led to the extraction of fragments having a mean molecular weight similar to that reported in the present paper.* Preliminary results showed that the diffusion constant was somewhat higher ($D_h = 5.7 \times 10^{-7}$; $D_m = 5.3 \times 10^{-7}$). The diffusion curves when reduced to normal coordinates deviated considerably from Gaussian curves, indicating a high degree of polydispersity. It is inherently unlikely, of course, that urea-bisulphite and strong alkali would have the same chemical effect on the wool fibre. Useful comparisons between different chemical treatments of wool cannot be made until chemical analyses of the different soluble products have been made.

V. ACKNOWLEDGMENTS

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* These extracts were kindly provided by Dr. F. G. Lennox.

RUMINAL FLORA STUDIES IN THE SHEEP

V. THE AMINO ACID COMPOSITION OF RUMEN BACTERIAL PROTEIN

By P. Holmes,* R. J. Moir,* and E. J. Underwood*

[*Manuscript received July* 6, 1953]

Summary

Fifteen amino acids were determined, by paper chromatography, on the protein preparations of each of two bulk samples of rumen bacteria from sheep fed under "dry" and "green" feed conditions.

Very similar patterns of amino acid distribution were found for the two samples and no outstanding differences were apparent between them and the values recorded in the literature for a range of microorganisms. The arginine, histidine, tryptophane, and glutamic acid contents were, however, higher in the rumen bacterial proteins and the isoleucine content somewhat lower than those recorded for other bacteria.

A comparison of rumen bacterial protein with whole egg protein is made which indicates that it is inferior as a source of leucine, threonine, and phenylalanine and markedly inferior as a source of methionine and isoleucine. Methionine and isoleucine are considered to be the "limiting" amino acids in the nutritional value of rumen bacterial protein.

These findings are discussed in relation to the utilization of nitrogen and the biological value of proteins to ruminants.

I. Introduction

Indisputable evidence that the bacteria of the rumen achieve considerable protein synthesis from non-protein nitrogen materials in the diet is now available from many sources. Even where the dietary nitrogen is entirely protein, appreciable conversion of this protein to bacterial protein is apparent from the work of Johnson *et al.* (1942), McDonald (1948*a*), and Moir and Williams (1950). A variable proportion of the nitrogen ultimately utilized by the ruminant is bacterial protein. For this reason several studies of the nutritive value of rumen bacterial protein have been made (Johnson *et al.* 1944; McNaught *et al.* 1950; Reed, Moir, and Underwood 1949). The last-named workers found the protein of their bacterial preparations, as judged by nitrogen-balance studies with growing rats, to be low in digestibility but relatively high in biological value. The biological value was, in fact, not significantly different from that found for casein fed under the same conditions, namely 78-80.

The biological value of a protein, determined under appropriately standardized conditions, provides a very useful indication of the overall nutritive value of the digestible portion of that protein to the young, growing, non-

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ruminant mammal. It does not, however, tell very much of the assemblage of amino acids present or of the specific amino acid deficiencies which may be limiting its usefulness to the animal. Actual amino acid analyses are valuable for this purpose. A beginning in this connection was made by Johanson, Moir, and Underwood (1949) and Reed, Moir, and Underwood (1949). These workers estimated, by the differential oxidation method of Lugg (1938), the cyst(e)ine and methionine contents of the protein of the mixed rumen bacteria samples used for the biological value determinations mentioned in the previous paragraph. They found the rumen bacterial protein to be surprisingly rich in cyst(e)ine, compared with almost all other food or microorganism protein studied, and moderately rich in methionine compared with many of them. The methionine content was, however, much lower than that of whole egg protein and slightly lower than that of muscle protein or casein (Block and Mitchell 1946). Since casein has been shown to be mildly deficient in methionine (Beadles *et al.* 1933; Kik 1938) and rumen bacterial protein has a biological value similar to casein it was tentatively concluded that methionine was the limiting amino acid. The possibility remained that other essential amino acids were also involved. These could only be revealed by much more complete studies in which all the essential amino acids were determined.

Several groups of workers have examined the protein of a range of microorganisms, including yeasts, actinomycetes, bacteria, and moulds, grown under a variety of cultural conditions. The results of these investigations have been critically assessed by Lugg (1949) and by Block and Bolling (1951) and will not be discussed at this point. Apart from the amino acid values of rumen material obtained from sheep fed urea as the entire N source (Loosli *et al.* 1949), and the cyst(e)ine and methionine values mentioned earlier, no data on the general amino acid make-up of the mixed bacterial protein from the rumen have, as far as is known, been published.

The methods and results of such studies are presented in this paper. They involve the quantitative determination, by means of paper chromatography, of 15 amino acids in the protein of bacteria obtained from the rumen of sheep selected to represent two types of feed conditions.

II. Materials and Methods

(*a*) *Collection and Preparation of Rumen Bacteria*

Details of the collection and preparation of the samples of rumen bacteria are given by Reed, Moir, and Underwood (1949). Two bulk samples were obtained from slaughter-house sheep; one from sheep which had come from dry feed conditions and the other from sheep brought from green grazing. These are referred to throughout as the "dry-fed" and the "green-fed" samples. Each sample was free from protozoa and feed particles and the dry material contained 47.6 per cent. crude protein (N × 6.25) in the "green-fed" and 50.9 per cent. crude protein on the dry basis in the "dry-fed."

(*b*) *Determination of the Amino Acids*

(i) *Protein Preparations.*—Representative 10-g samples were taken from each of the dried bacterial preparations and ground to pass a 0.5-mm sieve. "Whole" protein preparations were made from the ground samples by the method of Lugg (1939) as modified by Lugg and Weller (1944). Nitrogen determinations were made on the ground samples and on the "whole" protein preparations by the micro-Kjeldahl method.

(ii) *Protein Hydrolysis.*—For the estimation of tryptophane, alkaline hydrolysis of the protein preparations was carried out with 5N NaOH in sealed test tubes at 100°C for 24 hr. Following filtration the solution was neutralized with H_2SO_4 and made up to a volume of 20 ml. This neutralized hydrolysate was desalted prior to the chromatographic separation of the amino acids by the use of the electrolytic desalting apparatus designed by Consden, Gordon, and Martin (1947), except that the carbon anode was replaced with platinum (de Verdier and Agren 1948). Tests with standard tryptophane solutions gave a 91 per cent. recovery of tryptophane by this method. The final desalted solutions were then concentrated in a vacuum desiccator.

Acid hydrolysis was used for the rest of the amino acids determined. The protein preparations were hydrolysed with freshly distilled constant boiling HCl (5.7N) in sealed test tubes at 105°C for 24 hr. The quantity of protein preparation taken (200-500 mg) was such as to give a final concentration of approximately 5 mg N/ml with an acid : protein ratio of 10 ml/g. After filtering off the humin formed, the excess acid was removed by evaporation under reduced pressure. The resulting amino acid deposit was taken up in 5 ml of distilled water, slightly acidified to retain the less soluble amino acids in solution.

(iii) *Chromatographic Technique.*—The general methods adopted for the paper chromatographic separation of the amino acid constituents were based on the report of Consden, Gordon, and Martin (1944). For descending solvent movement air-tight galvanized iron tanks were used, the filter paper being suspended from stainless steel troughs. For ascending solvent migration, cylindrical glass museum jars were used in the manner of Williams and Kirby (1948). Whatman No. 4 filter paper was used throughout. Phenol was purified by the method of Williams and Kirby (1948), collidine by the method of Partridge (1948), and alcohols by fractional distillation. Water-saturated solvents were allowed to come to equilibrium until clear solutions were obtained at the chromatographic temperature. To avoid losses of amino acids associated with excessive heating in the presence of solvents (Fowden and Penny 1950) phenol was removed from the chromatogram by ether washing, collidine by drying in a current of air at 50°C, and alcohols by air-drying at room temperature. The dried papers were heated for a further 15 min at 100°C and the positions of the amino acids marked out under ultraviolet light (Phillips 1948).

Methionine, leucine, isoleucine, and phenylalanine were satisfactorily separated by the method of Work (1949) by uni-dimensional runs in the descending manner with water-saturated amyl alcohol as the solvent. The remaining amino acids were separated by two-dimensional runs using two solvent systems, namely the descending method of Consden, Gordon, and Martin (1944) with water-saturated phenol (1 per cent. NH_3) and collidine, and the ascending method of Boissonnas (1950) with phenol-water (7 : 3) and propanol-water (7 : 3).

(iv) *Amino Acid Estimation.*—Quantitative estimations of the amino acids were based on the reports by Fowden (1951) and Boissonnas (1950), who successfully adapted the ninhydrin reagent of Moore and Stein (1948) to a spectrophotometric estimation from chromatograms. Standard reference graphs, relating amino acid nitrogen to optical density, were drawn up for each amino acid by the use of standard amino acid solutions. The linear plot obtained was shown to be reproducible with an accuracy of ± 2 per cent. By reference to this standard curve quantitative estimations were obtained from the chromatograms with an accuracy assessed at ± 5 per cent.

III. Results

(*a*) *Whole Protein Preparations*

The moisture, total nitrogen, coagulable nitrogen, and protein nitrogen contents of the rumen bacteria samples, together with the nitrogen data for their "whole" protein preparations, are given in the paper by Reed, Moir, and Underwood (1949). The total nitrogen content of the bacteria from the sheep on dry feed is slightly higher (8.13 per cent.) than that of the "green-fed" sample (7.60 per cent.) but the reverse is true of their protein preparations. These are 8.78 and 9.62 per cent. for the "dry-fed" and "green-fed" samples respectively. Both bacterial samples were found to contain considerable amounts of non-protein nitrogen, and in the green-fed samples, of extractable carbohydrates which were removed during the separation of the protein preparations.

(*b*) *Loss of Amino Acids during Hydrolysis*

A variable destruction of amino acids during acid hydrolysis of protein materials, particularly where they contain carbohydrate, has been reported by many workers. In spite of this, the results of amino acid determinations on such materials are frequently presented as if no losses occurred.

The results obtained in the present study are similarly presented, not only to facilitate comparison with those of other workers but also because of the difficulties of assessing the magnitude of these losses. It is probable that no appreciable error is involved, by so doing, with threonine, alanine, phenylalanine, leucine, isoleucine, tryptophane, glycine, glutamic acid, and aspartic acid, as these are little affected by hydrolysis in the presence of carbohydrate (Block and Bolling 1951). With arginine, histidine, lysine, methionine, tyrosine, and valine, on the other hand, there is little doubt that results so presented are too low. Substantial hydrolytic losses have been obtained with these amino acids in an experiment conducted in this laboratory. The actual losses and the conditions of the experiment are being published elsewhere (Holmes 1953) since they do not relate directly to the rumen bacterial protein preparations under investigation. It is considered essential, however, that this point should be borne in mind for a proper appreciation of the results.

(c) *Amino Acid Composition of Rumen Bacterial Protein*

The results of the amino acid determinations on the protein preparations of the two samples of rumen bacteria, expressed as percentage amino acid N of protein N, are presented in Table 1, together with some representative figures on other bacteria obtained from the literature. The composition of a rumen material (Loosli *et al.* 1949) is also included. As urea is the sole source of dietary N, the protein present must be largely microbial, although salivary proteins are probably included. Many other individual amino acid figures could be quoted but those given are sufficiently complete to permit satisfactory comparison with our own results. It should be noted, however, that in all cases no allowance has been made for hydrolytic losses so that the results for the basic amino acids and for tyrosine and methionine are almost certainly too low. Certain other differences should also be mentioned. Our figures were obtained by chromatography on protein preparations of the rumen bacteria. The results of Camien, Salle, and Dunne (1945), and of Stokes and Gunness (1946) were obtained by microbiological assay of hydrolysates of whole washed bacterial cells. The data of Loosli *et el.* (1949) for rumen material were also obtained by microbiological assay and are stated as being "minimal."

TABLE 1

AMINO ACID COMPOSITION (PERCENTAGE AMINO ACID N OF PROTEIN N) OF THE PROTEINS OF RUMINAL AND OTHER BACTERIA COMPARED WITH WHOLE EGG PROTEIN

Amino Acid	Rumen Bacteria, "Dry-Fed"	Rumen Bacteria, "Green-Fed"	Rumen Material§	*Lactobacillus* spp. *	*Escherichia coli**	*Bacillus subtilis*†	Whole Egg Protein‡
Arginine	16·3	11·8	6·2	6·2-9·7	10·4	7·6	12·9
Histidine	4·8	6·2	2·4	2·6-4·1	3·3	2·5	3·6
Lysine	6·2	5·0	6·8	5·7-9·2	7·4	7·5	8·6
Threonine	2·9	2·6	2·9	2·4-3·6	2·8	2·6	3·6
Alanine	2·9	4·0	—	—	—	—	—
Phenylalanine	2·2	2·0	1·3	1·4-2·2	1·7	1·9	3·3
Leucine	4·5	4·0	3·3	3·4-5·0	5·0	5·1	6·1
Isoleucine	2·8	2·7	2·25	3·3-4·7	4·2	3·2	5·3
Valine	3·7	4·9	2·9	3·5-4·1	4·6	4·2	5·5
Tryptophane	1·4	1·2	0·5	0·3-0·5	0·6	0·5	1·3
Methionine	1·1	1·2	0·95	0·6-0·8	1·2	1·0	2·4
Tyrosine	2·5	1·9	—	—	1·4	1·5	2·2
Aspartic Acid	7·2	7·0	—	—	—	—	—
Glutamic Acid	11·5	12·1	—	5·8-6·9	—	—	—
Glycine	2·9	4·0	—	—	—	—	2·6

* Calculated from data of Camien, Salle, and Dunne (1945).

† Calculated from data of Stokes and Gunness (1946).

‡ Calculated from data given by Block and Bolling (1946).

§ Calculated from data of Loosli *et al.* (1949).

The two rumen bacterial samples exhibit, on the whole, very similar patterns of amino acid distribution. Of the essential amino acids the "dry-fed" sample contains appreciably higher amounts of arginine and lysine and smaller amounts of histidine and valine. The methionine contents of the two samples, namely 1.1 per cent. "dry-fed" and 1.2 per cent. "green-fed," are of the same order as the values found for the same material by Reed, Moir, and Underwood (1949), who used the "differential-oxidation" method of Lugg (1938). The figures given by these workers, namely 1.2 per cent. methionine N for the "dry-fed" sample and 1.5 per cent. for the "green-fed" indicate, however, a greater difference between the two samples.

A comparison of the rumen bacteria values with those of other bacteria given in Table 1 reveals no outstanding differences. The levels of lysine, threonine, phenylalanine, leucine, and valine are very similar in all the organisms studied, whilst methionine differs only in the low values recorded for the *Lactobacillus* spp. Arginine, histidine, trypotophane, and glutamic acid are appreciably higher in the isolated rumen bacterial protein than in the proteins of the other materials recorded, and isoleucine is slightly lower. The data obtained from these mixed populations provide further support for the general conclusions reached by earlier workers that the total proteins of microorganisms do not differ markedly in amino acid composition and that this composition is not greatly influenced by the conditions of growth.

IV. DISCUSSION

The finding from our own data and those of other workers that the bacterial protein, even when grown under widely varying conditions, is probably very similar in amino acid composition implies that rumen bacterial protein is relatively constant in composition for different conditions in the rumen. Since a varying but often high proportion of dietary protein and of non-protein N is converted in the rumen to bacterial protein and this protein becomes a major source of essential amino acids to the host animal it is of interest to examine its amino acid composition in relation to other proteins in the ruminant diet.

Whole egg protein can hardly be regarded as a normal source of N to the ruminant but it is of particular interest because it is almost perfectly utilizable for growth in the rat and is therefore regarded as possessing a practically ideal balance of essential amino acids for this purpose. Rumen bacterial protein, in comparison with this protein, is well supplied with arginine, histidine, valine, and tryptophane, is deficient in leucine, threonine, and phenylalanine, and is very deficient in methionine and isoleucine. In fact, the "limiting" amino acids, in the Mitchell and Block (1946) sense of that term, are almost equally methionine and isoleucine, as each of these has a percentage deficit of about − 50.

A comparison with more common sources of protein taken from the data of Holmes (1953), using the "total per cent. deficit" of essential amino acids in each case as described by Mitchell and Block (1946), indicates that rumen bacterial protein is inferior to vetch and lupin seed protein, of similar value to linseed and meatmeal, slightly superior to pea seed, and markedly superior to subterranean clover seed protein. The marked superiority to subterranean clover seed protein is of particular interest because this protein is even more severely deficient in isoleucine, methionine, and threonine than rumen bacterial protein itself, whereas vetch seed protein, for instance, is much better supplied with these three amino acids.

If it is assumed that a considerable proportion of dietary protein is converted to bacterial protein the comparisons just made suggest that, where the amino acid composition of rumen bacterial protein is inferior to that of a dietary protein, as with whole egg protein, the biological value (B.V.) of this dietary protein should be lower in the ruminant than in the rat. Support for this contention is given by the fact that Block and Mitchell (1946) report the figure of 96 for the B.V. of whole egg protein in the rat, compared with 86.7 obtained by Williams and Moir (1951) for this protein in growing lambs. Further, with linseed protein, in which the amino acid composition is, on our data and those of Holmes (1953), rather similar to that of rumen bacterial protein, the B.V. for growth in both the rat and the sheep are closely similar. Thus Block and Mitchell give the value of 78 for rats and Williams and Moir the value of 79.7 for lambs. Williams and Moir have also found no significant difference between the B.V. of linseed meal protein and subterranean clover seed protein in lambs, in spite of the much better essential amino acid make-up of the former (Holmes 1953). Moreover these workers obtained for urea a B.V. of 69 where this source of N constituted approximately half the dietary N of lambs, and Loosli *et al.* (1949) a B.V. of 46 where urea constituted the sole dietary source of N to lambs. Since urea supplies no essential amino acids it has obviously no biological value to rats. It is clear, therefore, that comparisons between the B.V. of dietary protein in ruminants and non-ruminants are of little value.

The utilization of dietary N in the ruminant depends upon the course of its metabolism within the rumen and is influenced by numerous factors such as its form and source, the level of intake, and immediate energy considerations (McDonald 1952; El-Shazly 1952; Synge 1952). Ingested N suffers a variable bacterial degradation to ammonia which may either be directly absorbed from the rumen (McDonald 1948*b*) and lost in whole or in part in the urine as urea or used as a source of N for protein synthesis by the ruminal bacteria where suitable carbohydrates are present. It is obvious that the ultimate value of any N source to the ruminant will be profoundly influenced by the degree to which one or other of these two processes of breakdown and synthesis predominate. Very little is yet known of the factors influencing either.

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GROWTH OF THE MOUSE COAT

II. EFFECT OF SEX AND PREGNANCY

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Summary

Cycles of hair growth on Naked mice are described. Effects of sex differences are small. Patterns of hair growth are markedly affected by pregnancy and lactation.

I. INTRODUCTION

Growth of the mouse coat is a cyclic phenomenon; during the growth phase the skin follicles form hairs which, when completed, are retained in the follicles. At the next growth phase the follicles form a new set of complete hairs. Hairs formed during previous growth phases may be shed during a growth phase, but Dry (1926) has shown that it is usual for a follicle to retain several completed hairs; as several hairs are retained by one follicle it is difficult to study growth in normal mice. The Naked gene has a dominant effect which results in the hairs breaking off at skin level just before, or soon after, the end of the growth phase (cf. Grüneberg 1951), so that mice carrying this gene are covered with hair only in parts of the body which are in the growth phase. Naked mice should, therefore, be very useful for studies of the characteristics of hair growth cycles in general, if it can be assumed that the Naked gene has no effect on the pattern of the hair growth cycles, since in them the presence of hair on the skin marks the passage of a growth cycle. In a later paper we will describe the results of comparisons made to check the assumption that Naked does not interrupt the normal growth cycle. In this paper some preliminary observations of the effect of sex and pregnancy on the growth cycles of Naked mice are given.

II. MATERIAL

The mice used in these studies are all from the NA stock, which was formed at the Genetics Laboratory, Animal Breeding and Genetics Research Organization, Edinburgh, by Dr. D. S. Falconer and A.S.F., assisted by Mrs. S. Sobey. The stock was formed by back-crossing Naked mice to the Strong A line of inbred mice for five generations. Thereafter the stock has been maintained by sib matings, extending over 7-10 generations, and can be considered to be very uniform genetically.

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III. Methods

Drawings of the mice were made once or twice a week, initially as shown in Figure 1*A*, *B*, later by the simpler method shown in Figure 1*C*, *D*. Drawings were made on a standard printed silhouette, and no check was made of body size. In order to make any statistical studies of the movement of hair bands it is necessary to transform these drawings onto a numerical scale. The scales are shown superimposed on the standard silhouette in Figure 1.

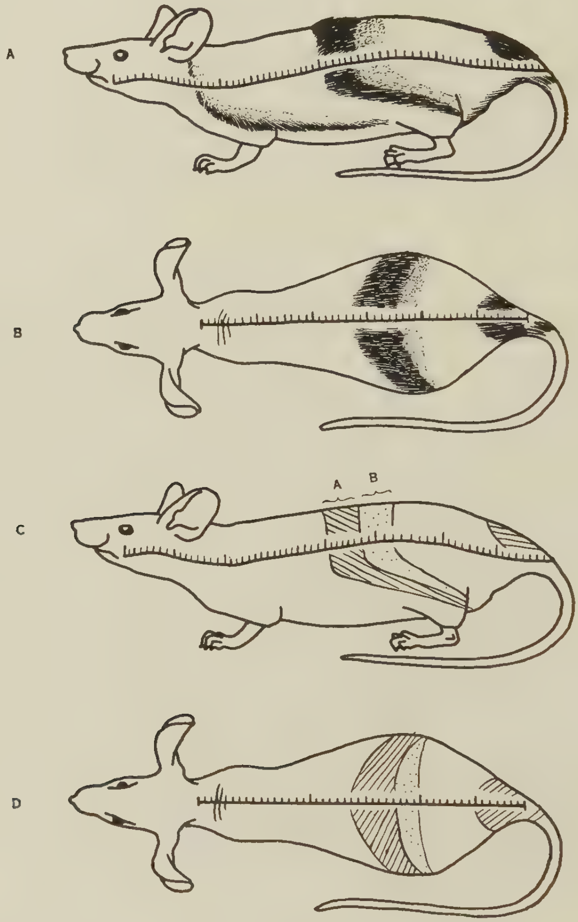

Fig. 1.—Standard silhouettes over which drawings were made. The original method of drawing, in which a copy of the structure of the hair bands was attempted, is shown in *A* and *B*. A schematized method of drawing was used later, involving the separation into a hair band (*A*) and a band of newly initiated hair growth (*B*). This is shown in *C* and *D*.

The transformations were effected by laying a celluloid sheet, on which the silhouette and scale were drawn, over each of the drawings; the positions of the front and rear edges of the hair bands were then read. The accuracy of the method was investigated, as described in Appendix I; the conclusion from these investigations was that the errors were sufficiently low to make the technique of value for the study of time changes in the bands, though the accuracy of plotting varied on different parts of the body.

IV. RESULTS

Naked mice typically carry two or three bands of hair spaced over the body. Drawings of a few mice showed that the bands of hair growth commence on the head and neck, move along the neck and over the head to form a band across the shoulders, which then moves along the back, terminating at the root of the tail. The drawings in Figure 2 are all of a single mouse, drawn at weekly intervals. A faint shadow extends beyond the caudal edge of the hair bands. This shadow is caused by the activity of hair follicles, which have only just begun their growth phase, and is the advancing edge of the hair band, which is moving from head to tail.

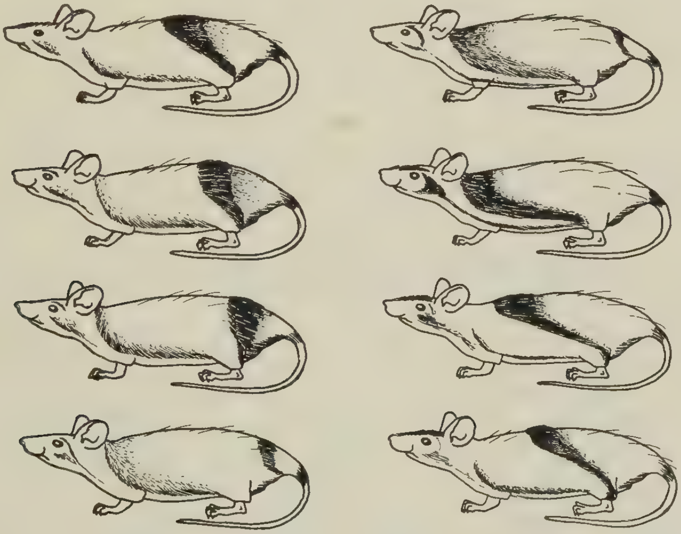

Fig. 2.—Drawings of an adult mouse, made at weekly intervals, demonstrating the pattern of movement of the hair bands.

(a) *Rate of Movement of the Hair Bands*

The rate of movement and width of a hair band change during the path from head to tail. The band moves faster along the back and sides than it does on the neck, shoulders, and near the tail. The bands on the neck, shoulders, and near the tail, are narrower than along the back. These points are illustrated in Figure 3, and in Table 1. The differences of width of the hair bands can be explained as an outcome both of the differences of speed of movement, and of differences of the length of the period during which a follicle is in the growth phase. The graphs of positions of hair bands plotted against time, shown in Figure 3, indicate that differences of length of the growth phase are small.

TABLE 1

RELATION OF WIDTH OF BAND TO ITS LOCATION

Location of Cranial Edge of Band	Mean Width of Band	Nos. of Bands
0-10	3·4	56
10-20	4·8	38
20-30	8·1	19
30-40	7·1	9
40-50	8·9	27
50-60	7·1	35
60-64	3·4	15

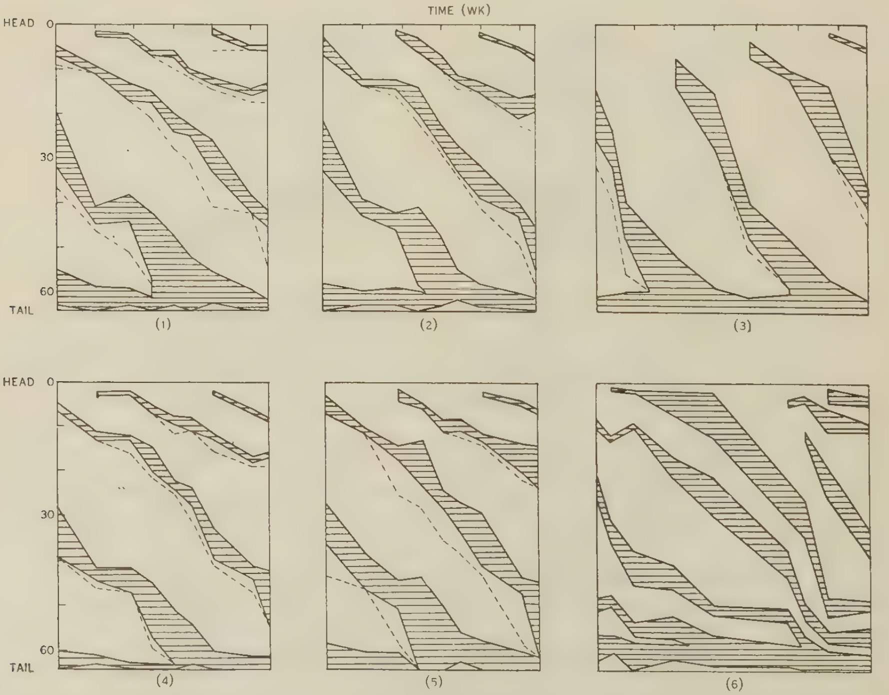

Fig. 3.—The positions of hair bands plotted against age, to show the pattern of movement and initiation of waves of hair growth. Each square is of a single mouse, (1) and (4), (2) and (5) are sister pairs. (1) and (2) were ovariectomized just before the beginning of observations. (3) and (6) are two unrelated, older females.

(*b*) *Similarity between Litter Mates*

The positions of hair bands in litter mates are very closely similar, which is not surprising, considering the genetic uniformity of the stock of mice used. In Figure 4 the similarity of sib pairs can be seen by comparing the graphs of hair band position. Divergence between sibs does occur, but at a very slow rate.

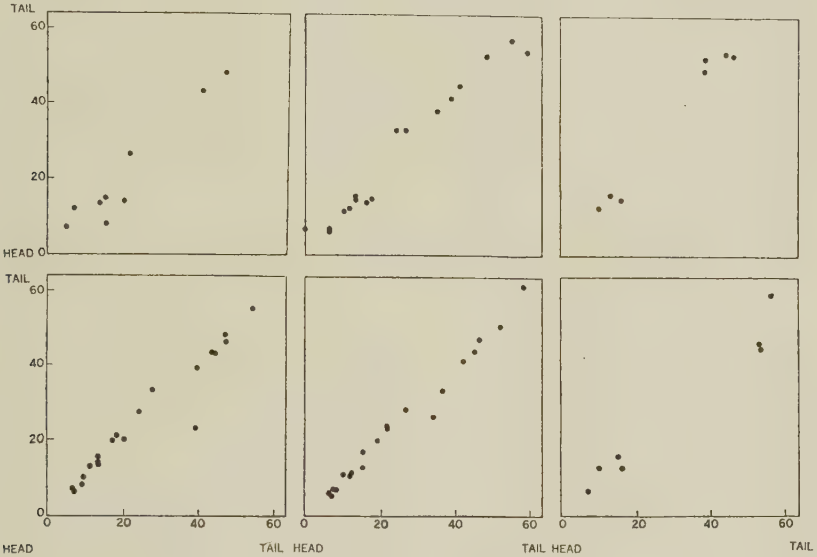

Fig. 4.—Positions of the front, or rear edges of hair bands are shown, for six pairs of sibs, plotted as a regression diagram, to illustrate the similarities between sibs.

(*c*) *Differences between Sexes*

Three types of comparisons were made to see if the hair cycle was the same in both sexes; between male and female litter mates (two pairs), castrated and entire males (one pair), ovariectomized and entire females (three pairs). No clear differences were detected between any of these pairs over the 4-5 wk during which drawings were made. A month after detailed observations were stopped the mice were examined and it was found that the ovariectomized females had wider hair bands than their controls, indicating that the rate of movement of the hair bands was increased by ovariectomy. The effect of sex, however, is quite small.

(*d*) *Effect of Pregnancy*

The first indication that pregnancy had an effect on hair growth was seen in a mouse which, although mated, did not become pregnant until it was 6-7 months old; prior to this the mouse had shown normal hair cycles. However, at 203 days it was found to be both pregnant and almost completely naked. Three days later a faint shadow appeared all over the body, except in the region where the preceding hair band had been located. Eight days later the

TABLE 2

HISTORY OF SECOND GROUP

Days Drawings Made (day No.)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
A								H											F			L				N			
B	L							H											F					L				N	
C	L															R	R	R											

F = fading of hair growth. H = reappearance of hair all over body. L = litter. N = completely naked. R = reappearance of hair growth as "reversed" band.

TABLE 3

HISTORY OF THE THIRD GROUP

Days Drawings Made (day No.)

Mouse	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
A	V.P.															F				L*				N									H						
B	V.P.															F					L*				N			H											
C	V.P.																	F			L				N														
D†	V.P.																																						

F = fading of hair cover. H = reappearance of hair all over the back. L = litter. N = completely naked. V.P. = vaginal plug. * = Killed. †No litter. Normal hair cycles.

whole body, except for the same narrow band, was covered with hair. On the ninth day the hair started to thin out, and 15 days after the appearance of the shadow a litter was born, and the mouse was again almost completely naked. This pattern of hair growth was so different from all our other observations that three groups of mice were observed to see if the aberrant pattern was correlated with pregnancy.

(i) *First Group.*—Drawings were made of six female litter mates; one was mated, the others being left unmated as controls. The controls showed normal hair cycles. Observations were not begun until some time after the female had been mated, and by the beginning of observations, she already had a litter. She differed strikingly from the other five mice, being naked except for a very narrow strip of hair. About 12 days after the birth of her next litter, a coat of dense, short hair appeared all over the back, except in the place previously occupied by the last hair band, which had now disappeared completely.

(ii) *Second Group.*—Three female litter mates were all mated, and had had 2-3 litters before detailed observations were begun. Their history is shown in Table 2.

Two (*A*, *B*) showed hair growth spreading all over the body 14 and 17 days before parturition, with subsequent fading until they became completely naked four days after parturition. The other mouse (*C*) was suckling a litter at the beginning of observations. This mouse was naked, and remained naked for 15-17 days after the birth of her litter. The animal then developed rather rudimentary bands, in which the new short hair appeared on the rear (cranial) edge of the band, instead of on the front (caudal) edge, as is usual. This phenomenon of "reversed" hair growth has been seen in other pregnant mice, and it will be studied in more detail.

(iii) *Third Group.*—Four virgin females (*A*, *B*, *C*, *D*) from different litters, and of different ages, were all mated to a single male, and then examined for vaginal plugs. All four showed plugs, but one (*D*) did not become pregnant. Their history is shown in Table 3. In the three pregnant females, the hair bands began to disappear 4, 5, and 3 days before the birth of the litter, and by 4 days after parturition they were completely naked. The fourth mouse (*D*) did not differ from other non-mated females in its pattern of hair growth. The females were separated from the male immediately after vaginal plugs were observed, and two of the litters (*A*, *B*) were killed shortly after birth. Thirteen and 7 days after parturition these two females showed hair growth spreading all over the back, except in one small region on one mouse. This was located where the previous hair band had disappeared. The skin was paper thin and transparent during the last days of pregnancy. A few days after parturition it became velvet-like, thick, and opaque, except in the region mentioned above. The skin of this region remained thin, and consequently was reduced to a deep furrow. The third female *C*, whose litter was left alive, remained naked until the end of observations; 26 days after parturition. These four mice are illustrated in Figure 5.

We can conclude that pregnancy has a distinct effect on hair growth. Summarizing, the hair bands become stationary as pregnancy progresses, and they begin to disappear approximately 5 days before the birth of a litter, resulting in the mouse becoming completely naked about 5 days after parturition. The renewal of hair growth after parturition followed one of two patterns:

(1) A new coat was formed simultaneously all over the body, except in regions which had been occupied by hair bands at the onset of the "pregnancy" effect.

(2) Approximately 2 wk after parturition rather rudimentary bands of hair were formed, which remained stationary and then disappeared. As noted above, the hair within these bands appeared to be initiated in a reverse order to normal (cranial to caudal instead of caudal to cranial).

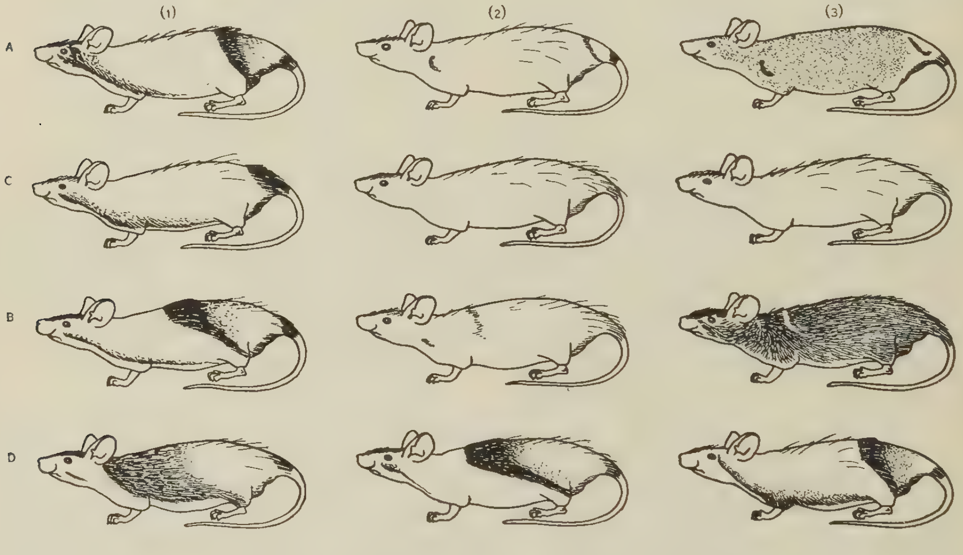

Fig. 5.—Effect of pregnancy on the third group (see text). The first column shows the four females before mating. The second column shows the mice 24-28 days later. The third column shows the mice 6-10 days later, to illustrate the resumption of hair growth.

V. Discussion

The density of hair cover in a mouse depends on (*a*) length of individual hairs, (*b*) diameter of the individual hairs, (*c*) number of hair follicles, (*d*) retention of completed hairs by the follicles, (*e*) frequency of waves of hair growth, and (*f*) the speed with which a wave of hair growth moves over the body. The Naked gene by stopping (*d*) allows us to observe (*e*) and (*f*) with comparative ease. We have shown that our methods allow fairly accurate estimates of these factors, and provided the statistical problems are solved, it is possible, using Naked mice, accurately to evaluate the effects of hormones etc. on the various determinants.

The cycling of hair growth is an intriguing example of a "biological" clock, and it is as such that we are primarily interested in its study. Durward and Rudall (1949) have shown, in the rat, using *iso*alloxazine to locate the waves of hair growth, that the timing of these cycles is regionally autonomous, and it is very probable that this regional autonomy also occurs in mice. Transplantation experiments are in progress to test this. The effects of ovariectomy and pregnancy indicate that some endocrine system has a regulating power over the timing of hair growth cycles.

Emmens (1942) in the rat, showed that "administration of androgens did not affect hair growth but oestrogens slowed it, more strongly in males than in females, in which more endogenous oestrogen is presumably already at work. Oestrogens also tend to cause bare patches to persist on the shaved area." These results suggest an explanation of the effects of ovariectomy and pregnancy. In our ovariectomized mice, the speed of movement of the hair band is faster than in the control females, and this is in accordance with Emmens' findings, since ovariectomized females will have a lower oestrogen level. In pregnant females the oestrogen level rises sharply towards the end of pregnancy and is paralleled by an inhibition of hair growth at about the same time. It is, however, difficult to reconcile the two types of resumption of hair growth with a simple removal of the inhibition.

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APPENDIX I

STATISTICAL TREATMENT

By HELEN NEWTON TURNER

To check the accuracy of plotting hair bands on standardized mouse silhouettes (left side and top), each of 10 mice was plotted three times, each diagram then being measured once. The number of bands present and their location on the mouse were studied on these diagrams, and because it seemed reasonable to expect that the accuracy of plotting might vary according to the location of the band, five arbitrary positions were defined:

(I) In front of ears;
(II) Commencing immediately behind ears;
(III) Commencing near centre of body;
(IV) Commencing above hind legs;
(V) Commencing at root of tail.

For the side view, measurements were made from a point near the tip of the nose, and on a line 1 cm from the back. For the top, measurements commenced just behind the ears; consequently bands at position I are missing from the top analyses. For each position, two or three measurements were available. The mean of these was taken as indicating the *location* of the band, and differences between consecutive pairs of measurements gave the *widths* of the two parts of the band, if present—the "full" band (*A*), and the "shadow" band (*B*). If in one drawing of a set of three, one band was omitted, as occasionally happened, the location measurement was omitted, but the width was entered as zero.

Standard deviations between repeated observations on the same mouse at the same position were then calculated, and are given in Table 4. The corresponding mean values are shown in Table 5.

TABLE 4

STANDARD DEVIATIONS OF BAND MEASUREMENTS (UNITS OF 1/16 IN.)

Position	Side			Top		
	Location	Width		Location	Width	
		A	*B*		*A*	*B*
I	1·33 (10)*	2·05 (12)	—	—	—	—
II	1·85 (17)	1·31 (18)	1·50 (16)	1·04 (5)	1·90 (6)	—
III	2·14 (10)	0·80 (10)	1·21 (8)	1·94 (10)	0·76 (10)	1·10 (8)
IV	1·14 (14)	1·34 (14)	—	1·34 (14)	1·21 (14)	—
V	0·78 (18)	1·20 (20)	—	0·58 (15)	0·92 (16)	—

* Numbers in brackets indicate the numbers of degrees of freedom on which each standard deviation was based.

The mean values for the locations of positions I-V on the side were 5.1, 15.4, 32.7, 52.3, and 60.8 units respectively. With the order of accuracy shown in Table 4, there would be clear discrimination even for positions intermediate between those chosen. The error of estimation of band width on position I is high, owing to the fact that this position was missed once in three drawings on each of two mice, although in one the width was considerable. For position II on the side, the standard deviation is also high in relation to the mean. The high value arises mainly from the contribution from one mouse, where the band on position II was missed. For the other positions, the standard deviations were sufficiently low to render the method valuable for the study of time changes in the bands. On the evidence of the present data, the side view gives more information than the top view; the standard deviations of measurements for top and side are similar, while not all bands present on the side are present on top. Table 5, for example, shows that only three of the 10 mice observed had bands in position II on the top view, whereas a band in this position on the side was present in nine out of the 10 mice. If the fact that a band fails to continue from the side over the top is important in any set of observations, then both side and top views should be included. Bands in positions III, IV, and V in the present series all continued across the top, so that a side view plus a top view from the shoulders forward should give a complete picture.

Table 5

MEAN VALUES OF BAND MEASUREMENTS (UNITS OF 1/16 IN.)

Position	Side			Top		
	Location	Width		Location	Width	
		A	*B*		*A*	*B*
I	5·1	3·0	—	—	—	—
	(6)[*]	(6)				
II	15·4[†]	1·5	3·7	4·4	2·9	—
	(9)	(9)	(8)	(3)	(3)	
III	32·7	3·8	4·5	18·0	3·9	3·3
	(5)	(5)	(4)	(5)	(5)	(4)
IV	52·3	5·2	—	37·5	5·2	—
	(7)	(7)		(7)	(7)	
V	60·8	3·7	—	44·9	4·2	—
	(10)	(10)		(8)	(8)	

[*] Numbers in brackets indicate the numbers of mice on which each mean is based.

[†] The scale for top measurements began behind the ears, that for the side near the nose; hence the differences in the location measurements between side and top.

Before concluding that the left-hand side view, as drawn, gives a satisfactory picture of the location and width of the bands, it was necessary to check the symmetry of the two sides. Ten further mice were included in this test, one drawing being made of each side. For positions II-V the between-side standard deviations were of the same order as those given in Table 4 for differences between repeated measurements on the same side. In one of the 10 mice only was there any serious discrepancy between the two sides; a "shadow" band (*B*) in position II was here shown as running over the back for the left-hand side drawing, but not for the right-hand side. A wide band extending over both positions III and IV in the same mouse was so poorly defined as to give bad agreement between the sides.

For position I, agreement between the two sides was poor. Only in four of the 10 mice were there bands of the same pattern and in the same location on both sides of the head in front of the ears. In three mice, bands were present on both sides but were different in pattern, while in the other three a band was shown on one side but not on the other.

The main use of the technique will be for studies in time changes of bands on the same mouse. The degree of asymmetry observed between left and right sides is not sufficiently great to invalidate the technique if used for such a purpose, but is sufficient to make it necessary for the observer to decide on one side and record it constantly.

The question of the optimum time interval between drawings for a study of time changes in the bands is now being investigated.

THE EFFECT OF METABOLIC INHIBITORS ON THE LOCAL ACTION OF OESTRONE AND OESTRADIOL-3,17β ON THE VAGINA OF OVARIECTOMIZED MICE

By P. J. CLARINGBOLD*

[*Manuscript received June* 24, 1953]

Summary

In the intravaginal Allen-Doisy test for oestrogenic activity potassium cyanide produced a significant increase in response to oestrone, but not to oestradiol-3,17β.

Sodium azide, 2,4-dinitrophenol, and sodium monoiodoacetate caused a reduction in response to both oestrogens studied.

Inhibitory dose response lines obtained with sodium monoiodoacetate in mice receiving maximal doses of oestrone or oestradiol-3,17β indicate greater variability of response to oestradiol-3,17β than to oestrone.

I. INTRODUCTION

While it has been known for some years that vaginal cornification in the rodent may be caused by local administration of oestrogens (see Emmens 1950, for review of the literature), the actual site and mode of action of the oestrogen in the epithelium have been the subject of speculation. It was thought that the effect of metabolic inhibitors, administered intravaginally, might help to elucidate this problem.

II. MATERIALS AND METHODS

A colony of 350 albino mice bred in this department were ovariectomized. Their management and use in tests were as described by Biggers (1951), tests being carried out fortnightly, with two injections in 24 hr. The oestrogens and inhibitors were administered together by the intravaginal route in phosphate buffer solution (pH 7.0, 0.1M), so that the total volume of two injections was 0.02 ml. The oestrone and oestradiol-3,17β were obtained from Organon Laboratories.

Standard procedures for probit analysis, as described by Finney (1952) have been employed throughout. At the start of this work it was thought probable that significant slope differences would be found, particularly when different oestrogens were being compared (Biggers and Claringbold 1953; Biggers 1953*a*). The estimation of relative potency for the comparison of activity is only justified if the dose response lines are parallel and if this is not so the median effective dose (M.E.D.) ratio test must be used (Biggers 1951). If the final

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TABLE 1

EFFECT OF POTASSIUM CYANIDE ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone						Oestradiol-3,17β					
Test	Control		Treated		M.E.D. Ratio	Test	Control		Treated		M.E.D. Ratio
	Dose (10^{-4} μg)	Response	Dose (10^{-4} μg)	Response			Dose (10^{-4} μg)	Response	Dose (10^{-4} μg)	Response	
1	1·0	0 : 20	1·0	4 : 19		1	2·0	9 : 20	2·0	10 : 19	
	2·3	3 : 20	2·3	8 : 20	1·83		3·0	12 : 20	3·0	11 : 19	1·23
	5·3	12 : 20	5·3	16 : 20	{1·12-2·98}		4·5	13 : 20	4·5	14 : 20	{0·52-2·90}
	12·2	15 : 20	12·2	17 : 20			6·8	17 : 20	6·8	16 : 20	
2	1·0	1 : 20	1·0	7 : 20		2	1·0	4 : 20	1·0	5 : 20	
	2·3	4 : 20	2·3	9 : 20	2·49		2·3	11 : 20	2·3	12 : 20	1·25
	5·3	13 : 19	5·3	17 : 19	{1·47-4·24}		5·3	12 : 20	5·3	12 : 20	{0·58-2·69}
	12·2	15 : 20	12·2	17 : 20			12·2	13 : 18	12·2	15 : 19	
3	1·0	3 : 20	0·4	3 : 20							
	2·3	10 : 18	1·0	10 : 19	1·91						
	5·3	12 : 20	2·3	17 : 19	{1·11-3·29}						
	12·2	19 : 20	5·3	17 : 20							
Mean slopes	2·29±0·27		1·71±0·24				1·37±0·34		1·27±0·34		
Common mean slopes	1·96±0·18						1·32±0·24				

Partitioning of χ^2

Source of variation	D.F.	χ^2	P	Source of variation	D.F.	χ^2	P
Parallelism				Parallelism			
Between mean slopes	1	2·68	0·2-0·1	Between mean slopes	1	0·05	0·8-0·7
Within mean slopes	4	0·99	0·95-0·9	Within mean slopes	2	1·04	0·7-0·5
Heterogeneity	11	22·40	0·05-0·02	Heterogeneity	8	5·87	0·7-0·5

	Oestrone	Oestradiol-3,17β
Common M.E.D. ratio	2·05 {1·24-3·38} $P=0·001$	1·24 {0·70-2·20}
Relative potency	2·28 {1·48-3·52} $P=0·001$	1·16 {0·64-2·11}

partitioning of χ^2 indicated no significant slope differences the relative potency estimate was computed, otherwise the more conservative M.E.D. ratio test was employed.

Response is recorded in the tables as a ratio of the number of animals positive to the number of animals per group. Standard errors of mean values follow a ± sign; fiducial limits of error ($P = 0.05$, except where otherwise indicated by subscript) have been enclosed in braces following the appropriate estimate; and M.E.D. ratios and relative potencies are given in their arithmetic form to avoid constant reference to the logarithmic base adopted in a particular test.

III. Results

(*a*) *Potassium Cyanide* (*Table* 1)

In the five tests reported 24 μg of potassium cyanide were injected with the oestrogen in buffer solution, three tests being carried out with oestrone and two with oestradiol-3,17β. Where oestrone was the oestrogen under test, a significant increase in response occurred on all occasions. The partitioning of χ^2 gives no evidence of any significant slope differences, and while the data obtained with oestrone show evidence of heterogeneity, this has been ignored since the total χ^2 for heterogeneity over all the tests reported in this paper is well within the range of random sampling variation ($\chi^2_{(54)} = 50.67$, $P = 0.59$).

(*b*) *Sodium Monoiodoacetate* (*Tables* 2 *and* 3)

The tests shown in Table 2 were based on the results of preliminary investigations and were designed for the purpose of comparing the inhibition produced by monoiodoacetate in mice receiving a maximal dose of (1) oestrone and (2) oestradiol-3,17β. The dose response lines have negative slopes and may be used to estimate the median inhibitory dose (M.I.D.), i.e. the dose required to prevent cornification in 50 per cent. of animals. Although equal amounts of oestrone and oestradiol-3,17β were given to both groups (20×10^{-4} μg), the expected response in the absence of inhibition is in each approximately 98 per cent. Under all conditions of administration oestradiol-3,17β has been found more variable in action than oestrone (Biggers and Claringbold 1953), with the result that the expected responses to doses of approximately 20×10^{-4} μg are equal (cf. Biggers and Claringbold 1953, Fig. 3).

Analysis of the results of Table 2 indicates that in order to produce 50 per cent. inhibition significantly more monoiodoacetate is required with oestradiol-3,17β than with oestrone. Partitioning of χ^2 indicates that the slope of the inhibitory dose response line for the latter is significantly steeper than for the former.

A simple factorial experiment (Table 3) was used to test the hypothesis that the activities of oestrone and monoiodoacetate are additive, i.e. to test whether the variability of response to oestrone is independent of the level of monoiodoacetate used. The results of the experiment have been fitted by means of a probit plane (Finney 1952), the regression equation involving the probit of response, the log dose of oestrone, the log dose of monoiodoacetate, and a constant. The goodness of fit of this equation was found to be satisfactory ($\chi^2_{(7)} = 2.68$, $0.95 > P > 0.9$). There is thus no evidence of departure from additivity and it may be concluded that the variability of response to oestrone is independent of the level of monoiodoacetate.

Table 2

EFFECT OF FOUR CONCENTRATIONS OF MONOIODOACETATE ON MAXIMAL CORNIFICATION PRODUCED BY 20×10^{-4} μg OF OESTRONE OR OESTRADIOL-3,17β

Twenty animals per group

Monoiodoacetate Dose (μg)	Animals Positive			
	Test 1		Test 2	
	Oestrone	Oestradiol-3,17β	Oestrone	Oestradiol-3,17β
10	17	11	18	13
20	14	7	11	10
40	3	6	7	5
80	0	4	6	5
*M.I.D. ratio:	2·00 {0·72-5·42}		1·71 {0·85-3·45}	
Common M.I.D. ratio	1·80 {1·02-3·19}			

Mean slopes:	
Oestrone	−2·47±0·39
Oestradiol-3,17β	−1·15±0·31
Common mean slope	−1·67±0·24

Partitioning of χ^2

Source of variation	D.f.	χ^2	P
Parallelism			
Between mean slopes	1	7·09	0·02-0·01
Within mean slopes	2	5·75	0·1 -0·05
Heterogeneity	7	6·40	0·5 -0·3

* Median inhibitory dose.

The data of this test have been analysed by alternative methods for purposes of comparison; the results of this investigation are published elsewhere (Claringbold, Biggers, and Emmens 1953). With these alternative procedures the same conclusions have been reached.

(*c*) *Sodium Azide* (*Table* 4) *and* 2,4-*Dinitrophenol* (*Table* 5)

In the tests reported 60 μg of sodium azide or 600 μg 2,4-dinitrophenol were injected with the oestrogen in buffer solution. The inhibitors always caused a significant lowering of response to the oestrogens. The partitionings of χ^2 show no significant slope differences.

TABLE 3

EFFECT OF COMBINATIONS OF LOCALLY ADMINISTERED DOSES OF MONOIODOACETATE AND OESTRONE

Twenty animals per group is constant; number positive tabulated

Oestrone Dose (10^{-4} μg)	Monoiodoacetate Dose (μg)			
	12·5	25	50	100
2	5	3	3	0
4	5	3	4	1
8	9	7	3	3
16	14	10	7	4

Mean slopes:

Oestrone: 1·17±0·24
Monoiodoacetate: −1·19±0·24

IV. DISCUSSION

Following the intravaginal administration of a dose of oestrogen, the changes which take place may be divided into two stages. In the first stage the oestrogen is absorbed by the epithelium and transferred to the final site of action, perhaps undergoing transformation on the way. If the dose is sufficient the second stage begins, involving morphological changes associated with greatly increased mitotic rate in the cells of the stratum germinativum. Metabolic inhibitors or activators may act on either of these stages; if acting at the first stage their effect may depend on the oestrogen used, whereas if acting at the second, their effect should be independent of the oestrogen used.

Evidence for the local conversion of oestrone to oestradiol-3,17β has been presented in an earlier paper by Biggers and Claringbold (1953), who have also pointed out the fundamental difference in variability of response to the local administration of these oestrogens. The differential action of cyanide with regard to oestrone and oestradiol-3,17β cannot be explained simply in terms of a local metabolic action taking place in the second stage, but must indicate that oestrone and oestradiol-3,17β undergo different changes in reaching their site of action.

Bullough and Johnson (1951) have studied the effect of various metabolic inhibitors on mitotic activity in adult mouse epidermis and found that cyanide, azide, monoiodoacetate, and 2, 4-dinitrophenol are powerful mitotic inhibitors acting in the antephase. The histological and cytological aspects of the vaginal

TABLE 4

EFFECT OF SODIUM AZIDE ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone						Oestradiol-3,17β					
Test	Control		Treated		M.E.D. Ratio	Test	Control		Treated		M.E.D. Ratio
	Dose (10^{-4} μg)	Response	Dose (10^{-4} μg)	Response			Dose (10^{-4} μg)	Response	Dose (10^{-4} μg)	Response	
1	1·0	2 : 20	1·0	1 : 20		1*			1·0	4 : 20	
	2·3	6 : 20	2·3	4 : 20	0·47 {0·25-0·91}				2·3	4 : 20	
	5·3	13 : 20	5·3	7 : 20			1·0	7 : 20	5·3	9 : 20	
	12·2	16 : 20	12·2	12 : 20			2·3	9 : 20	12·2	11 : 20	0·24 {0·09-0·67}
							5·3	13 : 20			
2	1·0	2 : 20	1·0	1 : 20			12·2	16 : 20	1·0	6 : 20	
	2·3	2 : 20	2·3	3 : 20	0·56 {0·37-0·83}				2·3	4 : 20	
	5·3	14 : 20	5·3	6 : 20					5·3	8 : 20	
	12·2	14 : 20	12·2	12 : 20					12·2	10 : 20	
Mean slopes 2·02±0·30			1·70±0·31				1·15±0·37		0·80±0·26		
Common mean slope 1·86±0·22							0·92±0·24				

Partitioning of χ^2							
Source of variation	D.f.	χ^2	P	Source of variation	D.f.	χ^2	P
Parallelism				Parallelism			
Between mean slopes	1	0·55	0·5-0·3	Between mean slopes	1	0·56	0·5-0·3
Within mean slopes	2	2·22	0·5-0·3	Within mean slopes	1	2·15	0·2-0·1
Heterogeneity	8	4·38	0·9-0·8	Heterogeneity	6	1·13	0·98-0·95
Common M.E.D. ratio 0·53 {0·38-0·75}				0·24 {0·09-0·67}			
Relative potency 0·49 {0·33-0·72}				0·23 {0·08-0·68}			

* A common M.E.D. for the two treated groups was obtained and tested against the control M.E.D.

TABLE 5

EFFECT OF 2, 4-DINITROPHENOL ON THE DOSE RESPONSE LINES OBTAINED BY THE LOCAL ADMINISTRATION OF OESTRONE OR OESTRADIOL-3,17β

Oestrone						Oestradiol-3,17β					
Test	Control		Treated		M.E.D. Ratio	Test	Control		Treated		M.E.D. Ratio
	Dose $(10^{-4}\ \mu g)$	Response	Dose $(10^{-4}\ \mu g)$	Response			Dose $(10^{-4}\ \mu g)$	Response	Dose $(10^{-4}\ \mu g)$	Response	
1	1·0	3 : 20	1·0	0 : 20		1	1·0	6 : 20	1·0	5 : 20	
	2·3	7 : 20	2·3	3 : 20	0·41		2·3	6 : 20	2·3	2 : 20	0·50
	5·3	12 : 20	5·3	8 : 20	{0·23-0·69}		5·3	12 : 20	5·3	9 : 20	{0·21-1·03}
	12·2	17 : 20	12·2	11 : 20			12·2	15 : 20	12·2	13 : 20	
2	1·0	2 : 15	1·0	0 : 15		2	1·0	7 : 20	1·0	2 : 20	
	2·3	4 : 19	2·3	2 : 20	0·48		2·3	9 : 20	2·3	5 : 20	0·32
	5·3	9 : 20	5·3	6 : 20	{0·24-0·94}		5·3	13 : 20	5·3	10 : 20	{0·13-0·78}
	12·2	16 : 20	12·2	10 : 20			12·2	16 : 20	12·2	11 : 20	
Mean slopes	1·91±0·31		1·98±0·36				1·18±0·26		1·25±0·27		
Common mean slope 1·94±0·23							1·22±0·19				

Partitioning of χ^2

Source of variation	D.f.	χ^2	P	Source of variation	D.f.	χ^2	P
Parallelism				Parallelism			
Between mean slopes	1	0·01	0·95-0·90	Between mean slopes	1	0·03	0·9-0·8
Within mean slopes	2	0·01	0·98-0·95	Within mean slopes	2	0·05	0·98-0·95
Heterogeneity	6	3·41	0·8-0·7	Heterogeneity	8	7·08	0·7-0·5

	Oestrone	Oestradiol-3,17β
Common M.E.D. ratio	0·44 {0·28-0·69}	0·40 {0·21-0·76}
Relative potency	0·44 {0·29-0·66}	0·41 {0·22-0·74}

response to oestrogens in mice have been discussed by Allen (1922) and Biggers (1953*b*), the first sign of oestrogen activity being division of the cells of the stratum germinativum. In the present work it would appear that azide, monoiodoacetate, and 2, 4-dinitrophenol are exerting their characteristic metabolic effect (inhibition of respiration in the first case, inhibition of glycolysis in the last two cases), resulting in inhibition of the morphological response. Bullough and Johnson (1951), however, found that cyanide acted similarly to azide whereas in the work described in this paper no inhibition occurred with the level of dosage used. In the present work the action of cyanide may be a specific enzymic effect. Heller (1940) showed that incubation of oestrone with liver mince resulted in inactivation of the oestrone unless cyanide was added, when oestrone was converted to oestradiol-3,17β with resultant increase in activity.

Further evidence has been presented as to the fundamental differences in activity and variability of oestrone and oestradiol-3,17β when administered by the intravaginal route. The slopes of the inhibitory dose response lines reflect the variability in response to the oestrogens, and indicate that oestradiol-3,17β elicits the more variable response. The fact that more inhibitor is required to produce 50 per cent. inhibition with oestradiol-3,17β than with oestrone demonstrates the greater relative activity of oestradiol-3,17β.

The present work, taken in conjunction with that of Bullough and Johnson (1951), indicates that oestrogens stimulate mitosis in the cells of the stratum germinativum, thus initiating the morphological process. Much remains to be done before a full understanding of the events leading up to this is possible.

V. Acknowledgments

I wish to express thanks both to Professor C. W. Emmens and Dr. J. D. Biggers for advice and criticism during the course of this work, which was assisted by grants from the Wool Industry Fund and the Commonwealth Bank of Australia.

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THE DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS IN FERTILIZATION AND EARLY SEGMENTATION

II. HISTOCHEMICAL STUDIES

By A. W. H. Braden[*] and C. R. Austin[*]

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Summary

Sections of rat eggs in various stages of fertilization and early cleavage were stained with buffered solutions of methylene blue or light green to show the distribution of basophilia and acidophilia respectively, or were treated by the Feulgen technique to show the distribution of desoxyribonucleic acid (DNA).

During fertilization the cytoplasm of the egg was evenly stained and showed weak basophilia and strong acidophilia. In the course of the first four cleavage divisions the basophilia decreased in the peripheral cytoplasm and increased in the perinuclear zone; the acidophilia retained its intensity, although in 8-cell and 16-cell eggs it was confined to the perinuclear zone.

The nucleoli of pronuclei and cleavage nuclei were very weakly basophilic and strongly acidophilic. In 4-cell, 8-cell, and 16-cell eggs a perinucleolar structure showing both intense basophilia and intense acidophilia was visible.

DNA was in general distributed as a shell about the nucleolus and as granules scattered through the nucleoplasm. In the full-grown oocyte high concentrations were evidently present in these locations. During fertilization a small amount of Feulgen-positive material was found in the early pronuclei, but none was seen in those of later stages. The concentration of DNA about the nucleoli increased greatly during cleavage, its distribution being similar to, but rather less extensive than, the perinucleolar structure just mentioned.

The results are discussed, particularly in relation to the histological findings of earlier workers and to the observations made by ultraviolet microscopy, which were described in a prior communication.

I. Introduction

In the first paper of this series (Austin and Braden 1953) an account was given of the distribution of nucleic acids in living rat eggs as determined by the absorption of ultraviolet radiation at 260 mμ. The method provided a reasonably reliable identification of nucleic acids, but could not be used to differentiate between ribonucleic acid (RNA) and desoxyribonucleic acid (DNA). To make such a distinction, it is necessary to apply histochemical procedures to sections of fixed eggs. This has been done and the results are described in the present communication.

Although many histological studies have been made on the mammalian egg during oogenesis, fertilization, and cleavage, in few have the more specific

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histochemical methods been employed. Gothie and Tsatsaris (1939) and Moricard (1949) reported observations on the distribution of DNA in the mouse oocyte, as shown by the Feulgen reaction. They found that the germinal vesicle of the full-grown oocyte did not stain, but in the oocyte just before meiosis the nucleolar membrane and the ring tetrads were Feulgen-positive. Alfert (1950) made quantitative estimates of the relative DNA contents of mouse egg nuclei. He found that the amount of DNA in the primary oocyte nucleus remains constant, so that its concentration falls as the nucleus increases in size during the growth of the oocyte. Each pronucleus in the egg during fertilization contains, according to Alfert, one-fourth the amount of DNA that is present in the primary oocyte nucleus, and each cleavage nucleus contains twice the amount of a pronucleus.

Available evidence on the general staining reactions of the nucleoli, particularly the pronuclear nucleoli, is conflicting. Earlier workers, such as Sobotta (1895), Rubaschkin (1905), Lams and Doorme (1908), Sobotta and Burckhard (1910), van der Stricht (1910), Lams (1913), and Mainland (1930), reported the presence of basophilic, non-staining, and eosinophilic nucleoli in pronuclei, the last two varieties predominating in later pronuclear development. Some nucleoli were described as having a strongly basophilic outer shell surrounding an acidophilic or non-staining interior. More recently Odor and Blandau (1951) observed uniform basophilic staining of nucleoli in early pronuclei, whereas many of the nucleoli in later pronuclei showed the basophilic shell just mentioned. In contrast to all these reports, Alfert (1950) saw no stainable nucleoli until the 4-cell and later cleavage stages, and stated: "Pronuclei and two-cell stage nuclei . . . . possess numerous Feulgen-positive shells which appear to be empty when judged by acid and basic staining." In a brief note, Flax (1951) described essentially the same finding from his investigations.

The results set forth in the present paper show a distribution of nucleic acids similar to that seen in living eggs by ultraviolet microscopy. In the pronuclei and cleavage nuclei the distribution of DNA was found to resemble that of the total nucleic acids. When treated under controlled conditions the nucleoli were always uniformly stainable, being faintly basophilic and strongly acidophilic. However, the staining reactions reported by the earlier workers could be reproduced by using the older histological methods.

II. Methods

Adult female rats, killed at the appropriate times after mating, provided full-grown oocytes, and eggs in various stages of fertilization and early cleavage. The ovaries, fallopian tubes, and uteri were removed from the animals and fixed for 18-24 hr in absolute alcohol containing 10 per cent. of glacial acetic acid. For the preservation of cell structures, and particularly the nucleoli, this fixative was found to be preferable to 10 per cent. formalin in saturated corrosive sublimate, Zenker-formol, and Flemming's weak osmic acid mixture.

Serial 8-μ sections of the paraffin-embedded tissues were stained either by the Feulgen technique as described by Stowell (1945) or by means of acid and basic dyes, over the pH range 4-8, according to the method of Dempsey, Wislocki, and Singer (1946). Methylene blue was used as the basic dye and light green as the acid dye. The dyes were employed at a concentration of 1×10^{-4}M in phosphate or sodium hydroxide-phthalate buffers of ionic concentration 0.05. After removal of the paraffin, the sections containing eggs were left in the dye solutions at 35°C for 20 hr, after which they were washed for 5 min in water and mounted in Apathy's mounting medium. The intensity of staining was estimated visually.

Sections of eggs were also stained with Mayer's haemalum and eosin. In some instances eggs were first stained by the method of Dempsey, Wislocki, and Singer (1946), inspected, washed with alcohol to remove the dye, and then stained with haemalum and eosin. By this means differences in the staining effected by the two methods could be observed in the same structures.

TABLE 1

BASOPHILIA AND ACIDOPHILIA OF RAT EGGS DURING FERTILIZATION AND EARLY CLEAVAGE

− to ++++ = Negative to intense staining

pH	Methylene Blue					Light Green					
	Nucleoli		Peri-nucleolar Material (8-16-cell stage)	Cytoplasm		Nucleoli			Peri-nucleolar Material (8-16-cell stage)	Cytoplasm	
	Pronuclear Stages	8-16-Cell Stages		Pronuclear Stages	8-16-Cell Stages*	Pronuclear Stages	8-16-Cell Stages	Uterine Muscle Fibres		Pronuclear Stages	8-16-Cell Stages *
4	−	−	−	−	−	+++	+++	+++	++++	+++	+++
5	−	±	+	−	±	++	++	++	++	++	++
6	±	±	++	±	+	+	+	+	+	±	±
7	±	+	++++	+	++	±	±	±	±	−	−
8	±	+	++++	+	++						

* Perinuclear cytoplasm only. The rest of the cytoplasm in these stages barely stained at all.

III. OBSERVATIONS

(*a*) *Staining with Acid and Basic Dyes*

Eggs during fertilization and early cleavage were stained with methylene blue or light green over the pH range 4-8; the results are set out in Table 1. Nucleoli of pronuclei during late primary, single nucleolus, and early and late secondary growth stages (Austin 1952) were found to be similar in their reactions; they were very weakly basophilic but showed evidence of a uniformly strong acidophilia (Plate 3, Fig. 13). The nucleoli of 4-, 8-, and 16-cell eggs also exhibited strong acidophilia (Plate 3, Figs. 14 and 15) and weak basophilia (Plate 3, Figs. 16-18). The basophilia appeared a little stronger than in the pronuclear nucleoli, but this increase was probably due to the shell of strongly basophilic material about the nucleoli of the cleaved eggs. The affinity for light green of the nucleoli in uterine muscle fibres in the sections containing 8- to 16-cell eggs was also noted in order to provide a comparison between early embryonic and somatic nucleoli. The two types of nucleoli were found to have closely similar reactions (Table 1). In eggs soon after sperm penetration, the metamorphosing sperm head and the nucleoplasm of the very early pronuclei were moderately basophilic.

Eggs stained with haematoxylin and eosin had some nucleoli which took up the haematoxylin strongly (Plate 3, Figs. 19 and 21), others which exhibited only a peripheral ring of stained material (Plate 3, Figs. 20 and 21), and others again which stained weakly with eosin. All types were seen in pronuclei during both primary and secondary growth phases. There was no obvious correlation between the size of the nucleolus and its staining reaction.

The pronuclei seen in Plate 3, Figures 19-21, are of particular interest. They had previously been stained with methylene blue by the method of Dempsey, Wislocki, and Singer (1946), when all nucleoli showed the faint uniform basophilia already referred to. The sections were then washed free of dye and stained with haemalum and eosin, and the result illustrated was obtained.

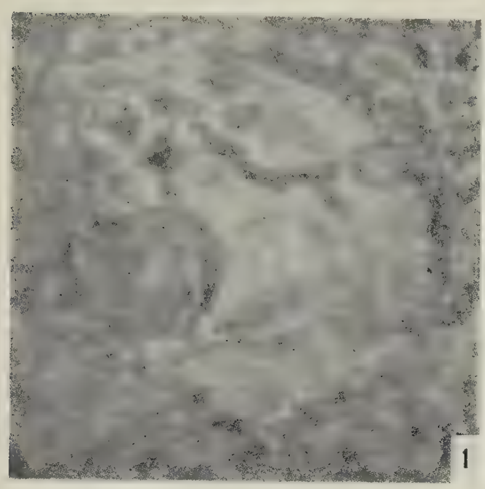
Aggregations of strongly basophilic material were regularly seen about the nucleoli in 8- to 16-cell eggs (Plate 3, Figs. 16-18) and, though less prominently, in 4-cell eggs. The perinucleolar material was also strongly acidophilic, slightly more so than the nucleoli (Plate 3, Fig. 15). The arrangement of the material was irregular but often resembled that of the perinucleolar material seen in living eggs by phase-contrast and ultraviolet microscopy (Austin 1953).

The cytoplasm of eggs in the fertilization stages was uniformly and weakly basophilic. In the early cleavage stages the basophilia was a little stronger, but was not evenly distributed; it was much more evident in the perinuclear zone than in the peripheral cytoplasm (Plate 3, Figs. 17 and 18). In the perinuclear zone the basophilia appeared to be associated with the cytoplasmic granules which were also gathered in this region. The egg cytoplasm in the fertilization stages stained evenly and strongly with light green (Plate 3, Fig. 13); in the cleavage stages the acidophilia was distributed, like the basophilia, almost exclusively in the perinuclear zone (Plate 3, Fig. 14).

(*b*) *Staining by the Feulgen Method*

The full-grown oocyte, just prior to the first maturation division, exhibited a ring of Feulgen-positive material around the nucleolus, and some scattered granules in the rest of the nucleus (Plate 1, Fig. 2). In the unfertilized tubal egg the only stainable structure was the metaphase chromosome group of the second maturation division (Plate 1, Fig. 4). The telophase chromosomes (Plate 1, Fig. 6), the sperm head undergoing metamorphosis (Plate 2, Fig. 8), and the recently formed male and female pronuclei were all stained by the Feulgen method. As the pronuclei increased in size the staining decreased in

DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. II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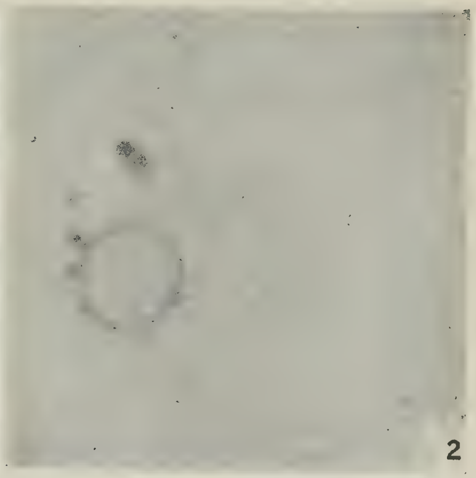
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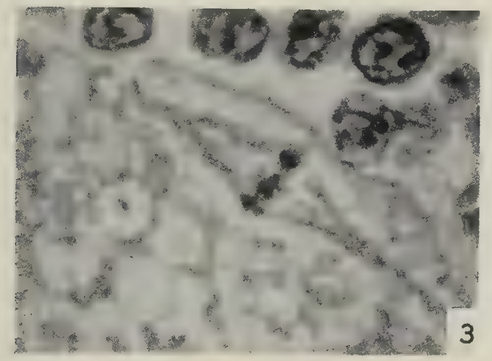
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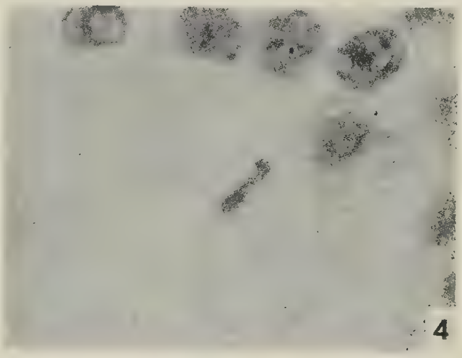
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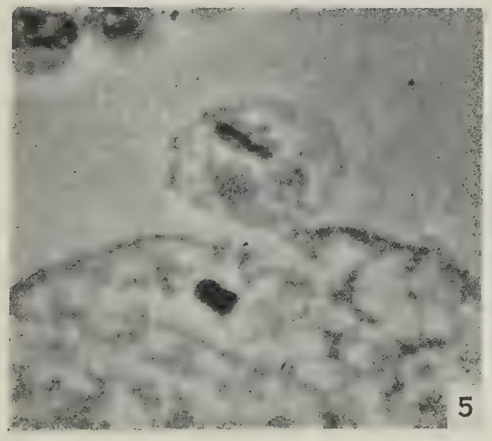
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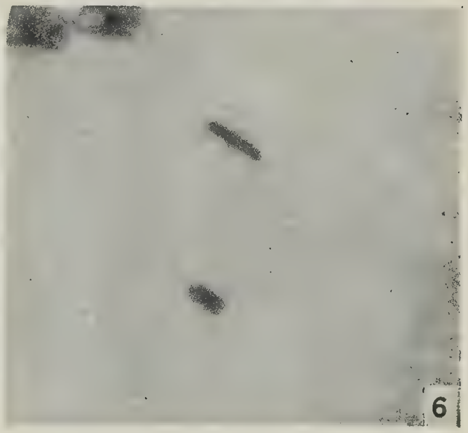
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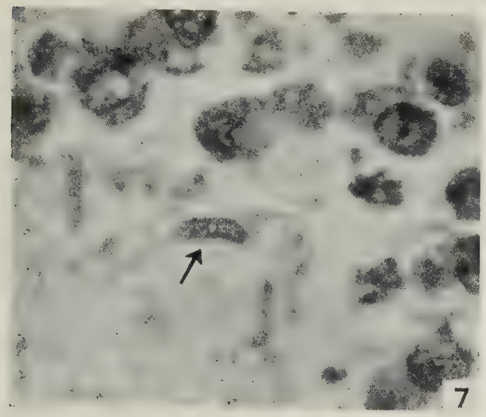
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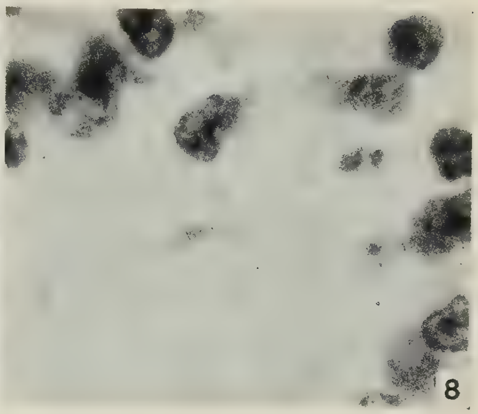
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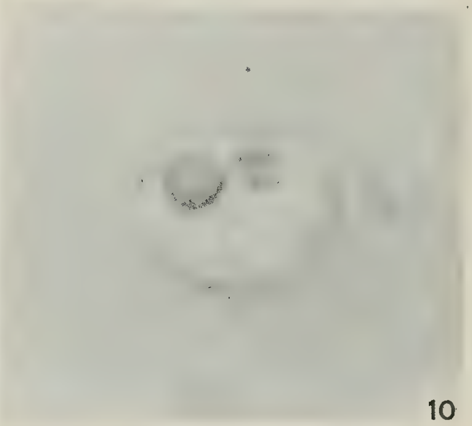
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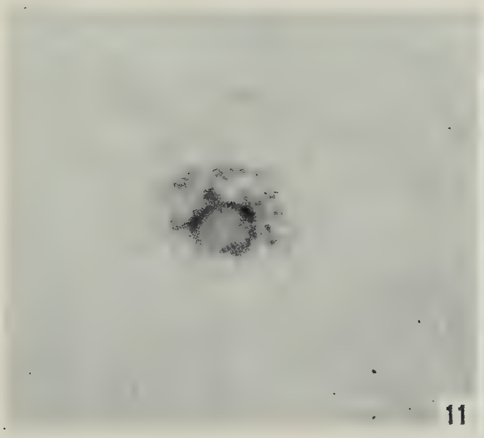
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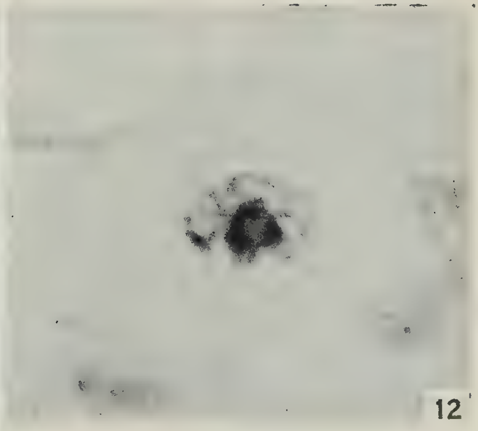
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DISTRIBUTION OF NUCLEIC ACIDS IN RAT EGGS. II

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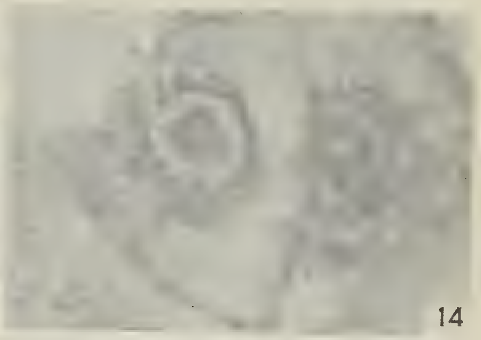
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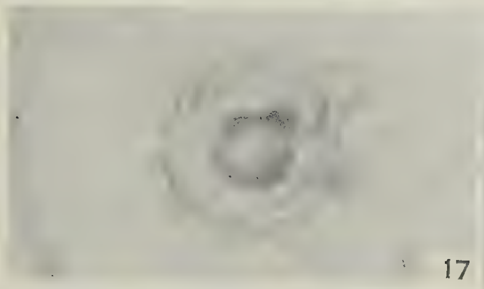
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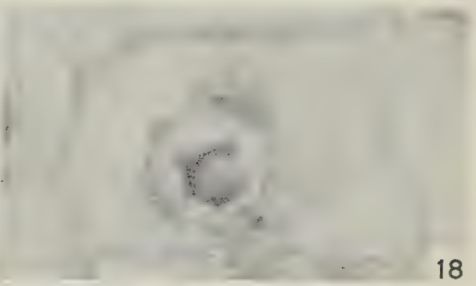
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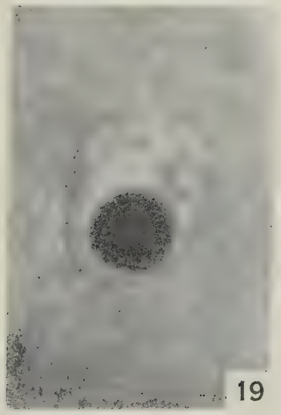
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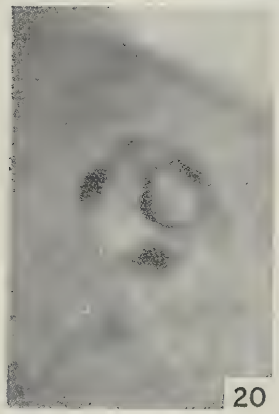
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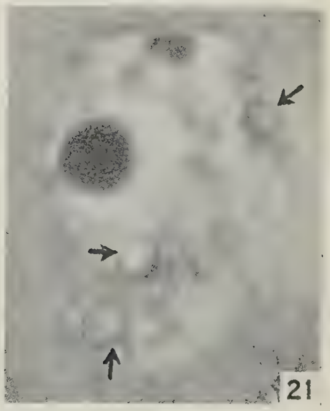
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intensity (Plate 2, Fig. 9) so that at the single nucleolus stage it was barely perceptible; in the later stages of pronuclear growth no staining could be detected.

After the first cleavage division the nuclei were again Feulgen-positive. The reaction was mainly evident around the nucleoli, though there were scattered stained granules throughout the nucleus (Plate 2, Fig. 10). The distribution of Feulgen-positive material was similar in the 4-, 8-, and 16-cell nuclei, but showed a progressive increase in amount with each stage of cleavage (Plate 2, Figs. 11 and 12). In the 8- and 16-cell eggs, the stainable material around the nucleoli was irregularly disposed and often appeared to correspond to the perinucleolar basophilic structure described in the preceding section, although it was rather less voluminous.

IV. Discussion

Results described in this paper show that, when stained under controlled conditions, the nucleoli of the rat egg during fertilization and early cleavage are uniformly very weakly basophilic and strongly acidophilic. These findings differ widely from those of earlier workers, who reported a variety of staining reactions in the nucleoli, some of which were said to be non-staining, others to be strongly basophilic, and others again to be composed of a pale central region with a strongly basophilic periphery. Results of this kind were obtained also in the present investigations when the older staining methods were employed. Indeed it was possible, by staining first with methylene blue under controlled conditions and then, after removal of the dye, staining with Mayer's haemalum, to reproduce both classes of results in the same nucleoli. From these observations it is concluded that the older staining methods, involving in most instances mordanting and differentiation, and with little control of pH or ionic concentration, are unreliable for the demonstration of basophilia and acidophilia.

It is clear from the work of Pischinger (1926, 1927), Dempsey, Wislocki, and Singer (1946), Singer and Morrison (1948), and many others (see Singer 1952 for review) that a much more precise chemical characterization of tissue proteins is obtained through a study of their affinity for acid and basic dyes under controlled conditions and over a range of pH. Proteins contain both acidic and basic groups, which, by their dissociation, give rise to positive and negative charges on the molecule. As the pH is lowered the basic groups are progressively dissociated and take up more and more of the acid dye, until dye is bound to all free basic groups. Similarly, as the pH is raised more of the basic dye is taken up by the acidic groups. Apart from the free basic and acidic groups in the protein molecule itself, charged groups are often present on substances conjugated with protein. The most important of these are nucleic acids and acid polysaccharides. It is concluded therefore that the nucleoli of the rat egg during fertilization and early cleavage contain very little nucleoprotein or acid mucoprotein, and that the protein present is predominantly basic in character. Nucleoli of mammalian nerve cells (Hyden 1943) and liver cells

(Lagerstedt 1949) are also made up in part of basic proteins, but in addition they contain ribonucleic acid and hence exhibit definite basophilia as well as acidophilia.

The cytoplasm of the egg, which also exhibits weak basophilia and strong acidophilia, is presumably similar in composition to the nucleoli, in that it has much basic protein and only a low concentration of nucleic acid. The increased basophilia in the perinuclear zone in 4- to 16-cell eggs may be simply the result of an aggregation of cytoplasmic nucleic acid, previously distributed evenly through the cytoplasm. Support for the present findings lies in the fact that the observed distribution and intensity of the basophilia correspond to the distribution and concentration of nucleic acids observed in living eggs by ultraviolet microscopy and described in the first paper of this series.

A positive Feulgen reaction, connoting the presence of desoxyribonucleic acid (DNA), was particularly evident immediately around the nucleoli of the full-grown oocyte and cleavage nuclei, in the sperm head during metamorphosis, in the chromosome groups of the second polar spindle, and in the nucleoplasm of the early pronuclei. This distribution corresponds closely to the regions of strongest ultraviolet absorption noted in living eggs. In resting nuclei DNA commonly shows a perinucleolar concentration, as may be seen from the descriptions of nerve cells (Hyden 1943), erythroblasts (Thorell 1947), mammary carcinoma cells and pollen grains (Koller 1947), rat liver cells (Lagerstedt 1949), and mouse eggs (Alfert 1950). The diminution of detectable DNA in the later pronuclei may well be due simply to the dilution of the nucleic acid, present in limited amount, as the pronuclei enlarge. In much the same way, during cleavage, a constant amount of DNA could be responsible for increased concentrations through its having to aggregate about progressively smaller nucleoli. These observations are therefore consistent with the findings of Alfert (1950) for the mouse egg.

It was reported by Austin (1953) and Austin and Braden (1953) that, in living 4- to 16-cell rat eggs, a perinucleolar structure, visible by phase-contrast microscopy and showing a strong absorption of radiation at 260 mμ in the ultraviolet, became progressively more evident. They concluded that the perinucleolar material was probably analogous to Caspersson's 'nucleolus-associated chromatin' (Caspersson 1950). In the present investigation a very similar structure has been found in fixed eggs and this showed a strong basophilia when stained under controlled conditions. In sections of eggs treated by the Feulgen method high concentrations of DNA could be detected immediately about the nucleolus. Although this Feulgen-positive material was arranged in such a manner that it resembled the strongly basophilic structure, it did not appear to be quite so voluminous. There is thus a likelihood that the DNA does not account for all the basophilia of the perinucleolar material, and that the balance is due to ribonucleic acid. As the structure also has a strong affinity for light green, it presumably contains in addition basic proteins; diamino proteins are described as constituents of the nucleolus-associated chromatin.

The present observations confirm and extend the investigations made in this laboratory on the cytology of fertilization and cleavage of the rat egg (Austin

1951, 1952, 1953; Austin and Braden 1953). Information has been obtained by varied methods, which have included the use of phase-contrast and ultraviolet microscopy for living eggs and the application of histochemical methods to fixed eggs. With appropriate fixation it is possible to obtain pronuclei in all major stages of development, as well as nuclei of the first four cleavage stages, showing essentially the same nucleolar number, form, and arrangement as that seen in living eggs by phase-contrast microscopy. Findings on the distribution of the nucleic acids in both living and fixed eggs have also been in good agreement.

From the observations of morphological changes during fertilization a broad picture is obtained of the development of the pronuclei. During this process the nucleoli are formed and increase in total volume and in number. Appearances suggest that the pronuclei are engaged in some important activity which may perhaps involve the formation of gene-modified "templates" to function later in the control of protein synthesis in growth. No chemical evidence for this theory has been found, however, and it seems clear from the absence of the nucleolus-associated chromatin that a synthetic activity of the kind conceived by Caspersson and his associates does not occur during fertilization. During the segmentation of the egg, both morphological and chemical data point to the development of part of the Caspersson system, for a structure analogous to the nucleolus-associated chromatin becomes increasingly apparent. The system, however, is evidently inactive during early cleavage, for the egg is neither increasing in size nor secreting. Moreover, the nucleoli contain little or no nucleic acid and the cytoplasmic nucleic acids do not show the large increase in concentration which is said to connote synthetic activity. Of interest in this connection is Alfert's (1950) report that a great increase in cytoplasmic basophilia was observable in recently implanted mouse embryos. Intense protein synthesis begins at this time and the cytoplasmic change may well signal the arousal to activity of a system previously at rest.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1-3

To assist in the identification of the structures, some of the figures showing distribution of Feulgen-positive material (Plate 1, Figs. 2, 4, and 6; Plate 2, Fig. 8) are accompanied by photographs of the same subject taken with the phase-contrast microscope (Plate 1, Figs. 1, 3, and 5; Plate 2, Fig. 7). Magnification throughout is × 1750.

PLATE 1

Figs. 1 and 2.—A full-grown oocyte shortly before the formation of the first maturation spindle. Feulgen-positive material is seen around the nucleolus and as granules in the nucleoplasm.

Figs. 3 and 4.—The second maturation spindle in a tubal egg, showing the strongly Feulgen-positive reaction provided by the metaphase chromosome group. The intensity of the reaction is about the same as in the nearby follicle-cell nuclei.

Figs. 5 and 6.—The second polar body soon after its extrusion. A strong Feulgen reaction is evident in the two groups of chromosomes and in the nearby follicle-cell nuclei.

PLATE 2

Figs. 7 and 8.—The sperm head in the ooplasm undergoing metamorphosis into the male pronucleus. The intensity of the Feulgen staining in this object is less than that of the nearby follicle-cell nuclei and has decreased distinctly from that shown by a sperm head before entry into the egg.

Fig. 9.—The distribution of Feulgen-positive material in an early male pronucleus is seen to be chiefly about the nucleoli and in the periphery of the nucleus. As the pronuclei grow, the intensity of staining decreases so that it cannot be detected with certainty when the pronuclei approach full development.

Figs. 10, 11, and 12.—The Feulgen staining of 2-, 8-, and 16-cell nuclei, respectively. Positively stained material is mainly perinucleolar, though much is distributed in granular form in the nucleoplasm. The increasing intensity of staining with decreasing nuclear size can easily be seen.

PLATE 3

Fig. 13.—A part-grown male pronucleus, stained with light green under controlled conditions, showing strong and uniform acidophilia in the nucleoli and cytoplasm.

Fig. 14.—A blastomere of an 8-cell egg, stained with light green under controlled conditions. Strong acidophilia is apparent in the nucleolus and in the perinuclear zone of the cytoplasm.

Figs. 15 and 16.—A nucleolus of an 8-cell egg which was stained first with methylene blue (Fig. 16), and then decolourized and stained with light green (Fig. 15), the staining being done under controlled conditions. The nucleolus itself shows faint basophilia and strong acidophilia, whereas the material attached to the nucleolus is both strongly basophilic and strongly acidophilic.

Figs. 17 and 18.—Blastomeres of 8-cell eggs stained with methylene blue under controlled conditions. Basophilia is most evident immediately about the nuclei and in the perinucleolar material.

Figs. 19-21.—Sections of eggs secured during pronuclear development and stained with Mayer's haemalum and eosin. Nucleoli can be seen which show the various forms of staining described by earlier workers. Two large and two small nucleoli show uniform intense staining; a large nucleolus has a strongly stained periphery and a pale centre, and there are three small nucleoli, indicated by arrows, which are stained peripherally only. These pronuclei had previously been stained with methylene blue under controlled conditions; all the nucleoli then showed only uniform faint basophilia. The sections were then decolourized and stained with haemalum and eosin.

AN INVESTIGATION OF POLYSPERMY IN THE RAT AND RABBIT

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Summary

A review is presented of published reports on polyspermy in mammals.

The cytology of living polyspermic rat and rabbit eggs, as seen with the phase-contrast microscope, is described. Polyspermic rat eggs have been seen in various stages of fertilization and early cleavage; observations on rabbit eggs are restricted to the later phases of pronuclear growth.

In the polyspermic rat egg during fertilization there is generally a close similarity between the male pronuclei; in the rabbit egg variations in pronuclear size are common.

In the polyspermic rat egg, the chromosome complements from the female pronucleus and the male pronuclei all take part in the formation of the first cleavage spindle. Polyspermic rat eggs at the 2-, 4-, and 8-cell stages are quite normal in appearance, except for the presence of the extra sperm mid-pieces.

The incidence of polyspermy under normal mating conditions as observed in 826 penetrated eggs from 87 rats was about 1.2 per cent. Among 69 pronucleate eggs from 12 rabbits the incidence was about 1.4 per cent.

When rats were mated at 10.0 p.m. (ovulation 12.0 midnight to 4.0 a.m.) the incidence in 353 penetrated eggs was about 3.7 per cent.; when mated at 3.0 a.m., the incidence in 315 penetrated eggs was about 7.9 per cent.; and when mated at 8.0 a.m., the incidence in 411 eggs was about 8.8 per cent. In 955 penetrated eggs from 75 immature rats, in which ovulation was artificially induced, the incidence of polyspermy was about 2.1 per cent. Altogether 112 polyspermic rat eggs were seen, including 109 dispermic and three trispermic.

When rabbits were mated at about the time of induced ovulation, 11 presumed polyspermic eggs (about 16.4 per cent.) were observed among 67 pronucleate eggs from 17 rabbits. The 12 polyspermic eggs seen in normal and delay-mated rabbits included nine probably dispermic and three doubtful eggs.

The results obtained from the rats are interpreted as showing that the susceptibility to polyspermy increases rapidly after ovulation, but approaches a limit within 2-3 hr. At this point, the number of eggs that become polyspermic is between one-quarter and one-third of all eggs having a chance of becoming polyspermic. Beyond 3 hr there is little further increase in susceptibility.

The probability that polyspermy gives rise to triploidy in rats, and the fate of the polyspermic rat egg, are discussed.

I. Introduction

From the intensive studies on fertilization which were made in the last quarter of the nineteenth century, it became apparent that, although only one

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sperm nucleus normally conjugates with the egg nucleus, more than one sperm may enter the egg and form a pronucleus. In some animals, notably among the insects, elasmobranchs, amphibians, reptiles, and birds, the entry of additional sperms, "polyspermy," was found to be a regular occurrence. The extra pronuclei in no way interfere with the subsequent development of the egg, unless a very large number of sperms is involved.

In other species, however, the penetration of the first sperm evokes the elevation of a membrane, or an alteration of the egg surface, which excludes other sperms. This block to polyspermy is not, however, fully efficient so that polyspermy does sometimes occur, and is then known as "pathological polyspermy." Its consequences are discussed by Wilson (1925); they vary according to the behaviour of the extra pronuclei. In *Ascidia* and the frog, only one of the male pronuclei conjugates with the female pronucleus, but whereas the polyspermic ascidian egg soon degenerates the frog egg may continue development and sometimes even reach the tadpole stage. The difference seems to depend on the fact that, in the frog, the mitotic figures from the extra pronuclei remain separate in the cytoplasm and more or less regular cleavages can take place. However, three or more blastomeres may result from the first cleavage and many of the blastomeres are binucleate, one nucleus being haploid and the other diploid. In the polyspermic sea-urchin egg, two or more male pronuclei may conjugate with the female pronucleus, giving rise to multipolar spindles and thus to multiple cleavage. Occasionally a dispermic egg will develop as far as a free-swimming larva, but most often there is early degeneration.

Information on polyspermy in mammals is scarce and confused, but several authors have declared it to be pathological in this class of animal (Lams 1913; Wilson 1925; Hartman 1934).

Rein (1883) stated that, in his attempts to obtain the fertilization *in vitro* of rabbit and guinea pig eggs, he had several times seen a number of sperms penetrate into ovarian eggs and there execute vigorous movements. He was not sure, however, if the eggs were still alive at the time. Rather similar are the observations of Lams (1913) and Blandau and Odor (1952). Lams described a guinea pig egg, the only "polyspermic" one among 108 eggs that he examined, saying that it was degenerate in appearance, with the chromatic material scattered throughout the vitellus. It contained several entire sperms, unchanged in form, in addition to the fertilizing sperm, the head of which was partially transformed into a pronucleus. Blandau and Odor report that they have seen, in freshly recovered rat eggs, accessory sperms penetrating the vitellus although it already contained a fertilizing sperm. According to these authors, the accessory sperms may enter and leave the vitellus a number of times, though none were seen to be retained within the vitellus. In another communication, Odor and Blandau (1949) found no polyspermic eggs in a total of 423 rat eggs which they had examined.

To a different category belong the four mouse eggs described by Sobotta (1895) among the 1140 fertilized eggs that he studied. Mice were found to mate a second time, 24 hr after the first mating, and in some of the eggs from these animals Sobotta saw objects resembling early pronuclei or sperm heads undergoing change in the egg cytoplasm. He considered that a second and later penetration of the eggs had occurred. Rubaschkin (1905) made rather similar observations on guinea pig eggs, in which he occasionally saw, besides the two normal pronuclei, a chromatic body staining strongly with haematoxylin or haemalum. These bodies closely resembled early male pronuclei. They were found also at later stages, such as the diaster stage of the first cleavage division. Rubaschkin thought they might indeed be accessory sperm nuclei, from a second and later penetration, but added that there was no certain evidence for this view. Kremer (1924) too recorded instances of a second and later sperm penetration in rat eggs, wherein he said it was more common than in mouse eggs. One of his figures shows a rat egg which has two well-formed pronuclei and, in addition, an apparently unchanged sperm head in the peripheral cytoplasm. One of the polyspermic eggs illustrated by Amoroso and Parkes (1947), obtained after inseminating rabbits with X-irradiated sperms, should be noted here for it also contained several unchanged sperm heads in the vitellus.

The observations of Gatenby and Hill (1924) suggest that polyspermy may be regarded as a normal process in *Ornithorhynchus.* This fact they consider to be consistent with the occurrence of physiological polyspermy in other megalecithal eggs, such as those of reptiles and birds.

Several authors have reported mammalian eggs with three pronuclei: two rat eggs, by Tafani (1889); one out of 36 pronucleated eggs in the cat, by R. van der Stricht (1911); two out of 11 pronucleated eggs in the cat, by Hill and Tribe (1924); one mouse egg, by Kremer (1924); one out of 71 pronucleated eggs in the ferret, by Mainland (1930); one rabbit egg, by Amoroso and Parkes (1947); one mouse egg, by Pesonen (1949); and three rat eggs, as well as one with four pronuclei, by Austin (1951). These authors considered the eggs to be polyspermic, except for Kremer, who thought that the phenomenon was due to the fertilization by one sperm of a binucleate egg, and Pesonen, who believed that the second maturation spindle, by completing its meiosis away from the surface of the egg, had given rise to two female pronuclei.

In a single instance, a cleaved polyspermic egg has been identified (Austin and Smiles 1948). This had two sperm mid-pieces in the cytoplasm and a small subnucleus in one of the blastomeres, but was otherwise normal in appearance.

From this brief review it can be seen that several different types of "polyspermy" have been described in mammalian eggs. Physiological polyspermy apparently occurs in *Ornithorhynchus.* Among the Eutheria, the phenomena described include eggs containing several unchanged sperms, others with three or more pronuclei at approximately equivalent stages of development, and others again in which early pronuclei were said to exist in the later stages of fertilization or in the blastomeres of 2-cell eggs. Little is known concerning the incidence of polyspermy and information on the fate of polyspermic eggs is lacking.

The investigations that are described in this paper were made with the purpose of obtaining a better understanding of the polyspermic mammalian egg, through studies on rats and rabbits. A preliminary account of the results has already been submitted (Austin and Braden 1953*a*): the present communication represents a full report on the work done.

II. METHODS

Randomly mated albino rats, and albino and cross-bred rabbits, were used in these studies.

In the adult rats in this colony, ovulation was found to occur principally between 12.0 midnight and 4.0 a.m., during the period of these investigations (Braden, unpublished data). For convenience, the day before ovulation will be referred to in the text as day 0, and the day on which ovulation occurs as day 1. The time of normal mating has been found to be during the late afternoon of day 0, but if oestrous female rats are placed with the males later, even as late as 9.0 a.m. on day 1, mating generally occurs.

Ovulation was induced in immature rats (35-50 days old) by the method of Rowlands (1944), except that B.D.H. "Serogan" and "Gonan" were used as sources of the gonadotrophin of pregnant mare's serum and chorionic gonadotrophin, respectively. When this procedure is employed, ovulation occurs mainly between 11 and 14 hr after the second injection (Austin 1951). The day of ovulation in the immature rat will be referred to in the text as day 1.

In rabbits, ovulation was induced by the intravenous injection of 50 I.U. of "Gonan"; it occurs about 10 hr after the injection.

Eggs were recovered from the rat fallopian tube by dissection under normal saline solution, and from the rabbit fallopian tube by flushing with saline. The eggs were examined with a phase-contrast microscope by the method previously described (Austin and Smiles 1948). Sometimes rat eggs under observation were fixed with aceto-carmine or 10 per cent. acetic alcohol and stained with methylene blue while compressed between coverslip and slide. Passage of the reagent to the egg is easily effected by applying drops of the solution to one side of the coverslip and a piece of filter paper to the opposite edge. The fluid is thus drawn under the coverslip.

III. OBSERVATIONS

(*a*) *Cytology of Polyspermic Rat Eggs*

In the present series, 109 dispermic and three trispermic rat eggs have been recovered and in these were represented most of the stages of fertilization and early cleavage. The earliest recorded is that shown in Plate 1, Figure 1; only two polyspermic eggs in this stage have been observed. Two sperm mid-pieces can be seen as they lie side by side in the superficial cytoplasm of the egg, and nearby are the two acrosomes. The nuclear parts of the sperm heads have undergone change and are at the stage when the nuclear material cannot be distinguished from the egg cytoplasm with the phase-contrast microscope.

Figures 2-5 show the pronuclei at various stages of development. In each of these eggs, two sperm mid-pieces are visible, and it is noticeable that the two male pronuclei are almost identical in size and structure. Rarely, polyspermic eggs are seen in which the correlation between the male pronuclei is not quite as close as this. In Plate 1, Figures 4 and 5, the later stages of pronuclear growth are represented; the latter shows the two male pronuclei in apposition.

Plate 1, Figure 6, illustrates a polyspermic egg which has been penetrated by two extra sperms and is therefore described as trispermic. All three male pronuclei can readily be discerned as well as some evidence of the three sperm mid-pieces. Unlike the great majority of polyspermic eggs, the male pronuclei in this trispermic egg are not identical in size and form, but the differences are not great.

Polyspermic rat eggs, recovered at later stages, have shown some of the phases involved in the formation of the first segmentation spindle. Plate 2, Figure 7, shows an egg which, when first seen, had three pronuclei in the terminal stages of their development. The egg was kept under observation and, within half an hour, three groups of chromosomes could be discerned in the cytoplasm; these are indicated in the figure. The egg was left for another hour in the hope that spindle formation would occur, but as little further change could be detected it was fixed and stained. The three groups of chromosomes could then be seen very clearly (Plate 2, Fig. 8). There is a suggestion of chromosome scatter which may have been due to an interference to spindle formation provided by the *in vitro* conditions. Another dispermic egg, which was observed to have three groups of chromosomes, was immediately fixed and stained, and then compressed very firmly beneath the coverslip. By this procedure a better view was obtained of the chromosome groups (Plate 2, Fig. 9).

Several polyspermic eggs have been seen in which only a single group of chromosomes could be discerned. The egg shown in Plate 2, Figure 10, is one of these, after it had been compressed by the method just described. Apart from this prophase chromosome group, no other basophilic material could be observed in the egg. Plate 2, Figure 11, shows a prophase in another polyspermic egg treated in the same way. No polyspermic rat eggs have been seen in which there was any indication that the chromosome groups from all pronuclei did not together take part in the formation of the first cleavage spindle. Polyspermic eggs with the anaphase or telophase stages of the first cleavage spindle have rarely been found, but one showing an early telophase is illustrated in Plate 2, Figure 12. This egg was fixed and stained but not squashed; the spindle fibres are perceptible in this preparation and the spindle as a whole appears normal.

A number of polyspermic eggs have been seen in the 2-, 4-, and 8-cell stages of cleavage. At these stages the diagnosis of polyspermy is more difficult than during pronuclear development for it depends entirely upon the recognition that two or more sperm mid-pieces are present within the cytoplasm of the blastomeres. Ofter "supplementary" sperms, that have penetrated the zona pellucida but have not entered the vitellus, can be seen in the perivitelline space during fertilization and segmentation. Quite frequently too, the heads become detached from these supplementary sperms and the headless mid-pieces then superficially resemble the mid-pieces of fertilizing sperms. The distinction can, however, be readily made by careful examination under the high power of the microscope. In the 2-cell egg, the mid-piece of a fertilizing sperm shows distinct signs of disintegration; much of its original opacity is lost, it may offer a granular appearance, and it is usually split for a variable proportion of its length. The disintegration becomes progressively greater in 4-cell and later stages of cleavage. By contrast, the mid-piece of a supplementary sperm remains uniformly smooth, opaque, and intact. Even if these structural differences are ignored, however, it is nearly always possible to decide definitely, after careful focusing, whether the mid-piece is in the cytoplasm or in the perivitelline space.

TABLE 1

TOTAL NUCLEAR AND NUCLEOLAR VOLUMES AND NUMBER OF NUCLEOLI IN NORMAL AND POLYSPERMIC 2-CELL RAT EGGS

	Egg Number	Total Nuclear Volumes (cu. μ)	Total Nucleolar Volumes (cu. μ)	Total Number of Nucleoli
Normal	1*a*	4990	744	8
	2*a*	5064	589	15
	3*a*	5952	584	7
	4*a*	7702	677	9
	5*a*	7484	819	5
	6*a*	4834	562	13
	7*a*	4582	702	10
	Total	40608	4677	67
	Mean	5801	668	9·6
Polyspermic	1*b*	5846	704	8
	2*b*	4796	607	11
	3*b*	6709	619	9
	4*b*	5106	632	15
	5*b*	7891	793	18
	6*b*	4752	572	14
	7*b*	5932	666	7
	Total	41032	4593	82
	Mean	5862	656	11·7

Aside from the presence of two or more mid-pieces within the cytoplasm, cleaved polyspermic eggs were found to be remarkably free from any evidence of abnormality. Polyspermic 2-cell rat eggs are shown in Plate 3, Figures 13 and 14. Parts of the two sperm mid-pieces can be seen in each egg, and the cytoplasm and nuclei are plainly very similar to those of monospermic 2-cell eggs. Plate 3, Figures 15 and 16, shows more clearly the two sperm mid-pieces as found in polyspermic 2-cell eggs. As is often the case, the two mid-pieces are apparently held together by a ring-shaped structure which is illustrated more clearly in Plate 3, Figure 16. This structure is commonly seen about the mid-piece of the sperm in normal 2-cell eggs, but is not invariably present.

With successive cleavage stages it becomes more difficult to discern the two sperm mid-pieces upon the presence of which the diagnosis of polyspermy depends. The danger is that an extra sperm mid-piece may be missed and not that a supplementary sperm in the perivitelline space may be taken for an extra penetrating sperm, for the difference between these two is very distinct in the later cleavage stages. Plate 3, Figure 17, shows the two sperm mid-pieces which were found in an otherwise normal 8-cell egg. They were much thinner than the sperms seen in the 2-cell egg (Plate 3, Fig. 16), apparently through loss of an outer granular layer; they were also much reduced in length.

In Plate 3, Figures 18, 19, and 20, are shown eggs which exhibit features that are unusual and may have a bearing upon some of the cases of "polyspermy" reported in the literature. Plate 3, Figure 18, shows a monospermic egg containing two female pronuclei which may have originated through the failure to extrude the second polar body. Quite often, segmented eggs are seen that have a small subnucleus in one blastomere in addition to the normal nucleus (Plate 3, Fig. 19). Sometimes the subsidiary nucleus is somewhat larger than this, but only once has an egg been seen which had two nuclei of about the same size and structure in one blastomere and a normal nucleus in the other (Plate 3, Fig. 20). Neither of these eggs was polyspermic.

Observations were also made on the total volumes of nuclei and the number and total volumes of nucleoli, in seven normal and seven polyspermic 2-cell eggs. The normal 2-cell eggs came from the same fallopian tubes as the polyspermic eggs and were measured so as to serve as controls. The method of measurement, together with data on normal values for segmented eggs, are described by Austin and Braden (1953*b*). The results are shown in Table 1: mean total nuclear volumes were 5801 cu. μ for the normal, and 5862 cu. μ for the polyspermic eggs, and mean total nucleolar volumes were 668 and 656 cu. μ respectively. The normal eggs had a mean of 9.6 nucleoli per egg, with a range from five to 15, and the polyspermic eggs a mean of 11.7, with a range from seven to 18. There was no significant difference in any of these values between normal and polyspermic eggs.

(*b*) *Cytology of Polyspermic Rabbit Eggs*

Although it is easy to see sperms in the perivitelline space of the living rabbit eggs, they can seldom, in the authors' experience, be detected in the cytoplasm. Consequently the diagnosis of polyspermy in the rabbit has had to rest solely upon the presence of more than two pronuclei. Normal eggs in the course of fertilization are shown in Plate 4, Figures 21 and 22; in each egg two well-formed pronuclei can be seen. In one egg the pronuclei are about the same size, whereas in the other they are distinctly dissimilar in size. Each pronucleus contained a few small nucleoli. Two presumptive polyspermic eggs are depicted in Plate 4, Figures 23 and 24. Each of these has three objects which are considered to be pronuclei, for they also contained a few small dark bodies like nucleoli and otherwise closely resemble the pronuclei in Plate 4, Figures 21 and 22. In one egg, however, two pronuclei are smaller than the third, whereas in the other egg two are larger than the third. The majority of the polyspermic rabbit eggs referred to later in this paper resembled one or other of these two types of eggs in approximately equal numbers. However, two eggs were found with six objects closely resembling pronuclei of various sizes. One of these eggs is shown in Plate 4, Figure 25; only four of the "pronuclei" can be seen, the other two being at a lower focal plane. So far as could be determined by careful focusing, there was no continuity between any of these "pronuclei" and thus no suggestion that the smaller ones were only lobules projecting from other "pronuclei."

(*c*) *Incidence of Polyspermy in Rats*

To determine the incidence of polyspermy under varying conditions, adult rats were mated and killed according to five different schedules:

(*A*) Female rats were mated under normal conditions, i.e. they were left with males overnight from 4.0 p.m., and those with copulation plugs the next morning were killed during the course of the day (day 1).

(*B*) Female rats were left with males for 1 hr only, between 10.0 and 11.0 p.m.; those with copulation plugs were killed during the next day (day 1).

(*C*) Oestrous female rats were selected about midnight by means of the vaginal smear and left with males for 1 hr between 3.0 and 4.0 a.m. of day 1; those with copulation plugs were killed during the course of the same day.

(*D*) Oestrous female rats, selected by vaginal smear, were left with males for 1-2 hr between 8.0 and 10.0 a.m. on day 1; those with copulation plugs were killed on the morning of day 2.

(*E*) Oestrous female rats, selected by vaginal smear, were left with males for 1 hr between 9.0 and 10.0 a.m. on day 1; those with copulation plugs were killed during the afternoon of day 4.

Observations were also made on the incidence of polyspermy in immature rats, and these were treated according to schedule *F*: Immature female rats received injections for the induction of ovulation and were left with adult males overnight from the time of the second injection (3.0-4.0 p.m., day 0); those with copulation plugs the next morning were killed at various times on days 1 and 2.

The numbers of rats used in each group, together with the principal observations made in these experiments, are set forth in Table 2.

The group of rats which were allowed to mate under normal conditions (schedule *A*) was made up of 87 animals, and these yielded a total of 826 eggs, of which 810 (98.1 per cent.) had been penetrated by sperms. Ten eggs, or 1.2 per cent. of the penetrated eggs, were polyspermic; in each of these one extra sperm had entered the vitellus. It was notable that in all the polyspermic eggs the two male pronuclei were closely similar in size and structure.

When rats were mated at 10.0-11.0 p.m. on day 0 (schedule *B*) a sudden increase in the proportion of polyspermic eggs was obtained: 3.7 per cent. of

TABLE 2

INCIDENCE OF POLYSPERMIC EGGS IN ADULT RATS MATED AT VARIOUS TIMES AND IN IMMATURE RATS AFTER INDUCED OVULATION

Schedule	Time of Mating	Time of Killing	Number of Rats	State of Eggs	Total Eggs	Total Penetrated Eggs	Polyspermic Eggs	% Polyspermic of Penetrated Eggs	Remarks
A	Day 0* 4 p.m.	Day 1* 11 a.m.-4 p.m.	87	Fertilization stages	826	810	10	1·2	All polyspermic eggs were dispermic
B	Day 0 10-11 p.m.	Day 1 11 a.m.-3 p.m.	39	Fertilization stages	366	353	13	3·7	All polyspermic eggs were dispermic
C	Day 1 3-4 a.m.	Day 1 10 a.m.-3 p.m.	32	Fertilization stages	323	315	25	7·9	Two trispermic eggs included
D	Day 1 8-10 a.m.	Day 2 9.30-10 a.m.	52	Fertilization and first cleavage stages	456	411	36	8·8	All polyspermic eggs were dispermic; one was degenerating
E	Day 1 9-10 a.m.	Day 4 2-5 p.m.	21	4-cell to 8-cell stages of cleavage	166	143	8	5·8	Polyspermic eggs were four 4-cell and four 8-cell; all dispermic
F	Day 0 4 p.m.	Day 1 and Day 2	75	Fertilization and first cleavage stages	1130	955	20	2·1	*Immature Rats*. One polyspermic egg was trispermic and one was degenerating

* Ovulation occurs between midnight on day 0 and 4 a.m. on day 1 in adult rats, and was induced between 2 a.m. and 6 a.m. on day 1 in the immature rats.

penetrated eggs. This figure is significantly different from the value obtained after normal mating ($P < 0.02$). There were 39 rats in this group and from them a total of 366 eggs was obtained, of which 353 (96.4 per cent.) were penetrated. As with the first group, all polyspermic eggs seen were dispermic.

In schedule *C*, 32 rats were mated between 3.0 and 4.0 a.m. on day 1, and these provided 323 eggs, of which 315 (97.5 per cent.) were penetrated. Twenty-five eggs, 7.9 per cent. of the penetrated eggs, were polyspermic, there being 23 dispermic eggs and two trispermic. This proportion of polyspermic eggs represents a significant increase above that observed after the 10.0 p.m. mating ($P < 0.05$).

All the penetrated eggs seen in schedules *A*, *B*, and *C* were undergoing fertilization; most of them were in the pronuclear stages.

The 52 rats mated, according to schedule *D*, at 8.0 to 10.0 a.m. on day 1, and killed 24 hr later, gave 411 penetrated eggs in a total of 456 (90.1 per cent.), including 36 polyspermic eggs (8.8 per cent. of penetrated eggs). This proportion of polyspermic eggs is not significantly different from the 7.9 per cent. noted in the rats mated at 3.0 to 4.0 a.m. (schedule *C*). No trispermic eggs were seen, all the polyspermic eggs having only one additional sperm within them. When recovered, the polyspermic eggs were in the terminal stages of pronuclear development (four eggs), in various phases of the first cleavage mitosis (13 eggs), or had undergone cleavage and were in the 2-cell stage (18 eggs). One undivided polyspermic egg showed nuclear fragmentation and was probably degenerating.

The polyspermic eggs seen in these four groups (schedules *A*-*D*) were well distributed among the rats. In general, only one polyspermic egg was found amongst the eggs from any one rat. However, one rat in schedule *A*, three in schedule *B*, three in schedule *C*, and six in schedule *D* provided two polyspermic eggs each. In addition, one rat in schedule *D* yielded three polyspermic eggs and another gave a total of four.

Observations were also made in these four groups of rats on the number of eggs which had sperms in the perivitelline space ("supplementary" sperms) in addition to one or more sperms in the vitellus. This material will form part of another communication but may be considered briefly here, since the number of eggs containing more than one sperm represents the total number of eggs in which polyspermy could have occurred under the conditions prevailing. It was found that, in the rats in schedule *A*, 22.5 per cent. of the penetrated eggs contained more than one sperm, while the figures for the other schedules were 27.8 per cent. for *B*, 30.4 per cent. for *C*, and 32.4 per cent. for *D*. The figures for the delay-mated groups do not differ significantly among themselves, but together represent a level which is very significantly different ($P < 0.001$) from the figure for the normally mated group in schedule *A*. The incidence of polyspermy in these groups was 1.23, 3.68, 8.37, and 8.76 per cent. respectively. The figure for group *C* was obtained by omitting the results from 10 rats which are included in the results shown in Table 2; these were omitted because supplementary sperms had not been counted in these animals. The incidence of actual in relation to possible polyspermy was calculated from the data just given and found to be 5.5, 13.3, 27.5, and 27.1 per cent. for the four groups *A-D* respectively (Table 3). A significant difference ($P < 0.05$) exists between 5.5 and 13.3 per cent., and between 13.3 and 27.5 per cent., but not between 27.5 and 27.1 per cent.

TABLE 3

NUMBER OF POLYSPERMIC EGGS, EXPRESSED AS A PROPORTION OF THE PERCENTAGE OF EGGS PENETRATED BY MORE THAN ONE SPERM, IN NORMAL AND DELAY-MATED RATS

Time of Mating	(1) Total Penetrated Eggs	(2) No. Eggs Penetrated by >1 Sperm	(3) Percentage of Eggs Penetrated by >1 Sperm	(4) No. Polyspermic Eggs	(5) Percentage Polyspermic Eggs	(6) (5) Expressed as Percentage of (3)
A, normal	810	182	22·5	10	1·23	5·5
B, 10.0-11.0 p.m.	353	98	27·8	13	3·68	13·3
C, 3.0-4.0 a.m.	227*	69	30·4	19*	8·37*	27·5
D, 8.0-10.0 a.m.	411	133	32·4	36	8·76	27·1

* The data for 3.0-4.0 a.m. mating in this table exclude results from 10 rats, included in Table 2, because supplementary sperms were not counted in the eggs from these animals.

In schedule *E*, 21 rats that had mated between 9.0 and 10.0 a.m. on day 1 were killed 3 days later, during the afternoon of day 4. From these animals 166 eggs were obtained, of which 143 (86.1 per cent.) had been penetrated. Eight eggs (5.8 per cent. of the penetrated eggs) were judged to be polyspermic; there were four 4-cell and four 8-cell eggs. Each had one extra sperm mid-piece in the cytoplasm of the blastomeres, but were otherwise quite normal in appearance. The sperm mid-pieces were often difficult to discern but once seen were easily distinguished from supplementary sperms lying in the perivitelline space, for they showed the advanced disintegration normally seen in the mid-pieces of fertilizing sperms, particularly in the later cleavage stages. The lower incidence of polyspermy found in this series may be misleading because some polyspermic 6-8-cell eggs may have been missed.

The 75 immature rats, treated according to schedule *F*, gave a total of 1130 eggs, of which 955 eggs (84.5 per cent.) were penetrated. There were 20 polyspermic eggs (2.1 per cent. of the penetrated eggs). This is not a significantly higher frequency than the 1.2 per cent. noted for the adult rats under normal mating conditions (schedule *A*). Nine of the polyspermic eggs were undergoing fertilization and 11 were in the 2-cell stage, including one egg which was trispermic. One of the polyspermic 2-cell eggs did not appear normal and was classed as degenerating.

(*d*) *Incidence of Polyspermy in Rabbits*

To obtain an indication of the frequency of polyspermy in rabbits under normal circumstances, 12 rabbits were mated and then killed 16-20 hr later. From these animals a total of 72 eggs was obtained; all of these eggs showed evidence of sperm penetration, and 69 (96 per cent.) had pronuclei. Only one egg (1.4 per cent. of the eggs with pronuclei) could be regarded as polyspermic, and this was a doubtful case since the "pronuclei" were ill-defined and may in reality have been pronuclear fragments.

Another group, consisting of 17 rabbits, received an intravenous injection of 50 I.U. of chorionic gonadotrophin and were mated 10 hr later, i.e. at about the time of the induced ovulation. These rabbits were killed 8-12 hr after mating and from them a total of 112 eggs was recovered. Eighty-two eggs (73 per cent.) showed evidence of sperm penetration, and 67 eggs (60 per cent.) were found to have pronuclei. There were 11 possibly polyspermic eggs (16.4 per cent. of the eggs with pronuclei), most of them (nine eggs) having three well-formed pronuclei. In each of the remaining two eggs, six closely grouped objects were found which resembled pronuclei of different stages. Among the eggs which had three pronuclei, the pronuclear size varied: in four eggs, one pronucleus was larger than the other two, in four other eggs, one pronucleus was smaller than the other two, and in one egg the pronuclei were graded in size. Observations on the incidence of polyspermy in rabbit eggs are summarized in Table 4.

TABLE 4

INCIDENCE OF POLYSPERMY IN EGGS FROM RABBITS AFTER NORMAL MATING AND AFTER MATING AT THE TIME OF OVULATION

	No. of Rabbits	Total No. of Eggs	No. of Penetrated Eggs	No. of Eggs with Pronuclei	No. of Polyspermic Eggs	Percentage*	Remarks
Normal mating	12	72	72	69	1	1·4	Polyspermic egg doubtful; possibly nuclear fragmentation
Late mating	17	112	82	67	11	16·4	Two polyspermic eggs doubtful; possibly nuclear fragmentation

* Polyspermic eggs as percentage of eggs with pronuclei.

IV. DISCUSSION

(*a*) *Cytological Features*

The observations described in this paper provide information on a number of features that characterize the polyspermic eggs of rat and rabbit, more especially the former. Polyspermic rat eggs have been seen in most phases of the formation and development of the pronuclei, in the formation and division of the first cleavage spindle, and in the 2-, 4-, and 8-cell stages of embryonic development. Observations on rabbit eggs have been limited to the later phases of pronuclear growth.

In the rat egg, diagnosis of the polyspermic state could be made with reasonable certainty because the two, or rarely three, sperm mid-pieces were regularly seen and often the extra acrosomes could also be discerned. A close correlation existed in the degree of development of the two male pronuclei and they were nearly always strikingly similar to one another in appearance.

Supposedly polyspermic rabbit eggs, on the other hand, may show either one small and two large pronuclei, or one large and two small pronuclei. Both these forms are referred to here as polyspermic although, without the evidence which the sperm mid-pieces would provide if they were clearly discernible, the diagnosis of polyspermy in rabbit eggs is uncertain. If the larger pronucleus is the male, as Amoroso and Parkes (1947) indicate, then eggs with one large and two small pronuclei may not be polyspermic. It is conceivable that, in such eggs, penetration by a single sperm has occurred after the second maturation spindle has moved inwards from the surface, because of delay in penetration. Completion of the mitosis would then give rise to two female pronuclei. This mechanism was considered by Pesonen (1949) to have been the mode of origin of the three pronuclei he had observed in a mouse egg, and a probable instance of the same phenomenon has been noted in the present series (Plate 3, Fig. 18). Actually the inward migration of the spindle is more likely to occur in the rabbit egg (Thibault 1951). On the other hand, the male pronuclei in polyspermic rabbit eggs may not always be similar in size or always larger than the female pronucleus as they apparently are in rat eggs. Some variation in the relative sizes of male and female pronuclei in normal rabbit eggs was in fact noted in the present series, and one trinucleate egg was seen in which the pronuclei were graded in size. Moreover, Amoroso and Parkes (1947) picture a rabbit egg with one large and two small pronuclei, indicating that they considered the large pronucleus and one of the smaller pronuclei to be male. Provisionally, it seems expedient to regard all the trinucleate rabbit eggs observed in this investigation as polyspermic.

Rather more problematic is the nature of the two rabbit eggs each of which was found to contain six apparent pronuclei of various sizes. This state may have arisen through the male pronuclei differing in their rates of growth, but it is also possible that the smaller "pronuclei" were produced by a process of lobulation and separation from a large pronucleus.

The evidence obtained indicates that, in polyspermic rat eggs, the female and both male pronuclei eventually give place in a normal way to groups of chromosomes which can be identified in the living eggs. The chromosomes then gather into a single prophase group from which the metaphase of the first cleavage spindle is formed. The steps involved in these processes, as seen in living monospermic rat eggs, have previously been described (Austin 1951). By fixing and staining polyspermic eggs in the appropriate stage, it has now also been shown that no chromatic material can be discerned in the vitellus, except that which composes the prophase or metaphase group of chromosomes. There seems to be no doubt, therefore, that all three pronuclei contribute their chromosome complements to the first segmentation spindle. Only one telophase stage has so far been seen, but to judge from this the mitosis of the first cleavage spindle may well proceed in a normal manner.

As with the pronuclear stages, the polyspermic 2-cell rat egg is quite normal in appearance, except that two sperm mid-pieces are present in the cytoplasm. Total nuclear volumes and total nucleolar volumes, as well as the total number of nucleoli, were closely similar in monospermic and polyspermic eggs. Quite often, the sperm mid-pieces in the polyspermic 2-cell egg both pass through a curious ring-shaped structure which is also commonly seen about the sperm in monospermic 2-cell eggs. It is possible that this ring arises during the process of cytoplasmic cleavage. When the constriction of the cytoplasm occurs at cell division, the sperm mid-pieces lying in the cytoplasm would tend to be brought together and then left with the ring-like relic still about them.

(*b*) *Previous Reports on Polyspermy*

Previous observations on polyspermic eutherian eggs are of a varied nature. The trinucleate eggs of Tafani (1889), van der Stricht (1911), Hill and Tribe (1924), and Mainland (1930) were almost certainly polyspermic, although the substantiating evidence of extra sperm mid-pieces in the vitellus was lacking. The trinucleate eggs reported by Kremer (1924) and Pesonen (1949) may also have been polyspermic, in spite of these authors' alternative suggestions. On the other hand, several of the instances recorded in the literature cannot properly be termed polyspermy, notably the grossly degenerate egg mentioned by Lams (1913). The eggs described by Sobotta (1895) and Rubaschkin (1905) may have suffered a second and delayed penetration as they suggest, but this seems unlikely. These authors may well have mistaken for early pronuclei the small basophilic objects of unknown nature which have been referred to in mammalian eggs, under such terms as "pseudochromosomes" or "corps enigmatiques," by Lams and Doorme (1908), van der Stricht (1910), van der Stricht (1911), Lams (1913), and Hill and Tribe (1924).

Alternatively, Sobotta may have mistaken for early pronuclei the small subnuclei often seen in apparently normal monospermic 2-cell eggs; an example in a rat egg is shown in Plate 3, Figure 19, and Blandau (1952) describes similar subnuclei in eggs from rats inseminated after ovulation. The subnucleus noted in a polyspermic 2-cell rat egg by Austin and Smiles (1948) was almost certainly another example of the same thing, and not the residue of a male pronucleus as they suggest. The apparent presence of unchanged sperm heads in the vitellus of pronucleate or 2-cell eggs, as reported by Kremer (1924), may have been due to artefacts arising during fixation or the cutting of the sections. Certainly, the evidence offered seems inadequate to support the idea that a second sperm could penetrate an egg so long after the first.

When the features of polyspermic rat eggs are compared with those of certain invertebrates and lower vertebrates, some major differences are seen to exist. The rat egg is unlike the ascidian or frog eggs in that the extra male pronuclei do not remain separate in the cytoplasm and give rise to separate spindles, but instead come into contact with the female pronucleus and all then contribute to the first cleavage spindle. In this particular, the rat egg is like that of the sea-urchin in which the chromosomes from all pronuclei may become involved in the first cleavage spindle. Under these circumstances a multipolar spindle is formed in the sea-urchin egg, the number of poles being related to the number of extra centrosomes introduced by the sperms. No suggestion of multipolar spindles was seen in polyspermic rat eggs—an observation which raises some doubt about the function of centrosomes in the cleavage of fertilized rat eggs.

In polyspermic eggs of both frogs and sea-urchins, the first cleavage may result in the formation of three or more blastomeres; there is no indication of such a process with rat eggs. Binucleate blastomeres are commonly found in the cleavage stages of polyspermic frog eggs but have not been seen in polyspermic rat eggs. One rat egg with a binucleate blastomere was indeed seen in the present series (Plate 3, Fig. 20), resembling in this way a bat egg described by van der Stricht (1910), but it contained only one sperm mid-piece and so could not have been polyspermic.

(*c*) *Increased Incidence of Polyspermy after Delayed Mating*

The incidence of polyspermy in rat and rabbit eggs under normal mating conditions was low, about 1.2 and 1.4 per cent. respectively, which is consistent with the small number of previously reported cases in these and other species. In immature rats after normal mating the incidence of polyspermy (2.1 per cent.) was not significantly higher than in adult rats, which seems to indicate that the artificial induction of ovulation does not appreciably influence the resistance of the eggs to the entry of more than one sperm into the vitellus. The increase in the incidence of polyspermy that occurred as a result of delayed mating in adult rats and rabbits is striking, both for the rapidity of increase and for the high incidence finally reached. An increase in incidence of about three times (to 3.7 per cent.) occurred in rats mated at 10.0 p.m., which is about 2-6 hr before the expected time of ovulation. This is remarkable because the time of penetration of eggs in these animals could hardly differ appreciably from that obtaining after a normal mating, and it does not seem possible for the eggs to have undergone any change. However, the sperms at the site of fertilization would presumably be fresher and more vigorous at the time of ovulation, in rats mated at 10.0 p.m., than in rats after normal mating. This is likely because with normal mating the sperms would have to spend several more hours in the female tract before the eggs reach the tube. The greater vigour of the sperms may, therefore, be the main reason for the sharply increased incidence of polyspermy observed with 10.0 p.m. mating. In support of this contention is the fact that the proportion of rat eggs containing more than one sperm is about the same after the 10.0 p.m., 3.0 a.m., and 8.0 a.m. matings (27.8, 30.4, and 32.4 per cent.) and that these three values are on a level which is significantly greater than that after normal mating (22.5 per cent.).

When rats were mated at 3.0-4.0 a.m. the incidence of polyspermic eggs was found to be 7.9 per cent., and when mated at 8.0-9.0 a.m., the incidence was 8.8 per cent.; the figures do not differ significantly from one another.

These values are a little more than twice that obtained for the 10.0 p.m. mating, and between six and seven times that for normal mating. As with the 10.0 p.m. mating, the high incidence of polyspermy is to some extent referable to the greater vigour of the sperms, but this explanation does not seem adequate to account for the increase in incidence of polyspermy beyond that noted for the 10.0 p.m. mating. Moreover, after the 3.0 a.m. and 8.0 a.m. matings, the number of eggs that became polyspermic represented a larger proportion (27.5 and 27.1 per cent., respectively) of the total number of eggs that theoretically could have become polyspermic under the circumstances prevailing. These considerations point to the occurrence of some change in the egg itself that renders it more susceptible to polyspermy. The susceptibility increases rapidly after ovulation but apparently reaches a limit early, at a point where between one-quarter and one-third of the eggs that have a chance of becoming polyspermic, actually do so. It is shown elsewhere that when rats were mated at 3.0 a.m. the penetration of most of the eggs occurred 2-3 hr later than in rats normally mated (Braden and Austin, unpublished data). The change responsible for the increased susceptibility to polyspermy must therefore take place principally in this short period, because no further increase in the incidence of polyspermy was found with the 8.0 a.m. mating. A rapid rise in susceptibility of a similar nature is known in some sub-mammalian forms: the change in *Asterias* eggs, for example, was found to be such that over 80 per cent. became polyspermic when inseminated after standing in sea-water for less than 2½ hr (Clark 1936).

A striking contrast with the findings presented in this paper is provided by the recently published observations of Blandau (1952) who artificially inseminated 78 rats in four groups: before ovulation and at 3-5, 6-8, and 9-12 hr after ovulation. Blandau describes a number of interesting abnormalities in eggs undergoing fertilization and in the 2-cell stage, which could be imputed to the entry of sperms into aging eggs. However, in not one of the 777 penetrated eggs that he examined had more than one sperm entered the vitellus. The absence of polyspermy from Blandau's material seems remarkable and an adequate explanation for it is difficult to find; it may perhaps be referable to the use of artificial insemination in place of natural mating.

If the diagnosis of polyspermy in the rabbit is correct, and this question has already been discussed, the incidence after mating performed at the time of ovulation (16.4 per cent.) is much higher than with normal mating and higher even than that seen after delayed mating in rats. This is consistent with the fact that, compared with rat eggs, a much larger proportion of rabbit eggs contains supplementary sperms in the perivitelline space and the numbers of supplementary sperms are also larger (Braden and Austin, unpublished data). There would therefore be much greater chances of polyspermy in rabbit than in rat eggs. The increase in polyspermy after delayed mating in rabbits may provide an explanation for the occurrence of polyspermy when rabbits were inseminated with X-irradiated sperms (Amoroso and Parkes 1947), because irradiation of the sperms was observed to cause a delay of 3-10 hr in the penetration of the eggs.

(*d*) *Fate of the Polyspermic Embryo*

The increased incidence of polyspermy resulting from delayed mating may have important practical and theoretical implications. So far, however, there appears to be no information on the fate of these embryos. In the present study, polyspermic eggs have been found to develop at least to the 8-cell stage without showing any noticeable abnormality. The lower incidence of polyspermy observed in 4- and 8-cell eggs may not have any significance because diagnosis becomes progressively more difficult as cleavage proceeds, and it is possible that some polyspermic 8-cell eggs were missed.

The evidence obtained indicates that polyspermy may be an important source of triploidy in rats, for both male pronuclei apparently contribute to the formation of the first cleavage spindle. On the other hand, nuclear size, nucleolar number, and nucleolar volume in polyspermic 2-cell eggs do not differ significantly from normal 2-cell eggs, and it is commonly known that triploid cells often have larger nuclei and more numerous nucleoli than diploid cells. Decisive evidence depends on the determination of the actual number of chromosomes on the first, or on any subsequent, cleavage spindle, and attempts are at present being made here to obtain this information. If, as seems likely, polyspermy (dispermy) does in fact give rise to triploidy, then the problem of the fate of the polyspermic embryo becomes the problem of the fate of the triploid embryo. A triploid mouse embryo at 9½ days of development has been recorded by Fischberg and Beatty (1951), but there is as yet no certainty that triploid embryos can survive to birth (Beatty 1951).

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Addendum

Since this paper was submitted for publication, the authors have been able to make analysable squash preparations of the prophase or early metaphase chromosome groups on the first cleavage spindle in four dispermic rat eggs. Estimates of the chromosome numbers were: 57 ± 5, 60 ± 5, 60 ± 5, and 60 ± 3 ($2n = 42$). These results clearly indicate the presence of a triploid number of chromosomes on the first cleavage spindle and further support the conclusion drawn in the paper, that polyspermy (dispermy) in rats probably gives rise to triploidy in the embryo.

Explanation of Plates 1-4

A_1 and A_2: acrosomes detached from penetrating sperm heads. *Chr.*: chromosome groups. S_1, S_2, and S_3: sperm mid-pieces. ♂, ♀ : male and female pronuclei.

Plate 1

Polyspermic rat eggs at various stages of fertilization. Phase contrast. × 700.

Fig. 1.—An egg seen shortly after entry of the two sperms into the vitellus. The sperm heads have undergone some degree of change and are in the stage at which they are not discernible in the cytoplasm by phase-contrast microscopy. Both sperm mid-pieces and both acrosomes can be seen.

Figs. 2-5.—Eggs in stages of pronuclear development. Both sperm mid-pieces can be seen in three of these pictures. The close similarity in development of the two male pronuclei is striking.

Fig. 6.—A trispermic egg. The three male pronuclei and the three sperm mid-pieces can be discerned.

Plate 2

Polyspermic rat eggs at stages in the formation and division of the first cleavage spindle.

Figure 7 by phase-contrast microscopy; remainder by direct illumination of stained preparations.

Fig. 7.—This egg was first observed in the terminal stages of pronuclear development, and is shown here at a stage soon after the three pronuclei had given place to three groups of chromosomes. × 700.

Fig. 8.—The same egg after it had been kept *in vitro* for about 2 hr and had then been fixed and stained. There are three distinct groups of chromosomes and there was no other strongly basophilic material in this egg. × 700.

Fig. 9.—Squash preparation of a polyspermic egg showing three groups of chromosomes. × 1000.

Figs. 10 and 11.—Squash preparations of polyspermic eggs showing single groups of chromosomes, evidently in the prophase of the first cleavage spindle. Figure 10 × 1000, Figure 11 × 1500.

Fig. 12.—Early telophase spindle in a polyspermic rat egg. × 1100.

PLATE 3

Polyspermic eggs in early cleavage stages (Figs. 13-17) and some anomalies seen in monospermic eggs (Figs. 18-20). Phase contrast.

Figs. 13 and 14.—Polyspermic 2-cell eggs. Two sperm mid-pieces are easily seen in each egg. Both eggs appeared otherwise to be quite normal. × 700.

Fig. 15.—The two sperm mid-pieces in 2-cell eggs are often found arranged in the manner shown in this picture: they appear to be bound together by a ring-shaped structure. × 700.

Fig. 16.—The two sperm mid-pieces in another polyspermic 2-cell egg are seen more clearly to pass through the ring-shaped structure. × 3000.

Fig. 17.—The two sperm mid-pieces in a polyspermic 8-cell egg. They are reduced in diameter and lack the granular mitochondrial sheath. × 1500.

Fig. 18.—A monospermic egg showing one male and two female pronuclei. × 700.

Fig. 19.—A monospermic 2-cell egg with a small subnucleus in one blastomere in addition to the normal nucleus. × 700.

Fig. 20.—A monospermic 2-cell egg having two nuclei of similar size and appearance in one blastomere; the other blastomere had a single nucleus of the same form and dimensions. × 700.

PLATE 4

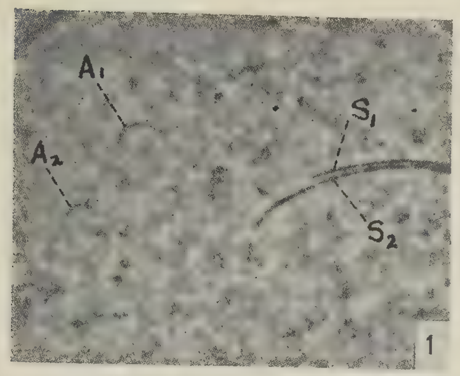
Pronuclei in normal and polyspermic rabbit eggs. Phase contrast. × 700.

Figs. 21 and 22.—Rabbit eggs obtained from an animal after normal mating. In the first, the pronuclei are about the same size, but, in the second, one is nearly twice as big as the other.

Figs. 23 and 24.—Eggs with three pronuclei. These were obtained after delayed mating. In one (Fig. 23), two pronuclei are smaller than the third, while in the other (Fig. 24), two are larger than the third.

Fig. 25.—An egg with six possible pronuclei; two small ones are not visible at this focal plane.

POLYSPERMY IN THE RAT AND RABBIT



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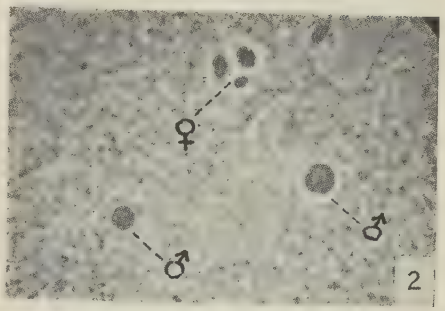

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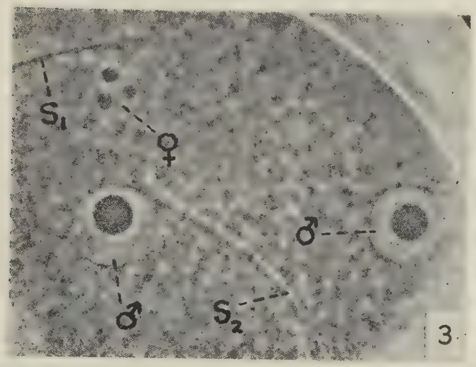

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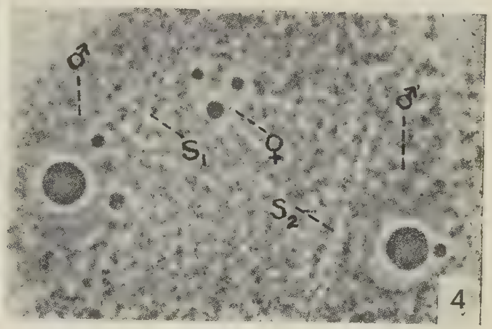

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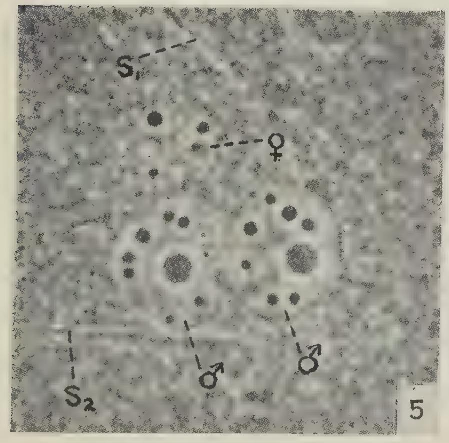

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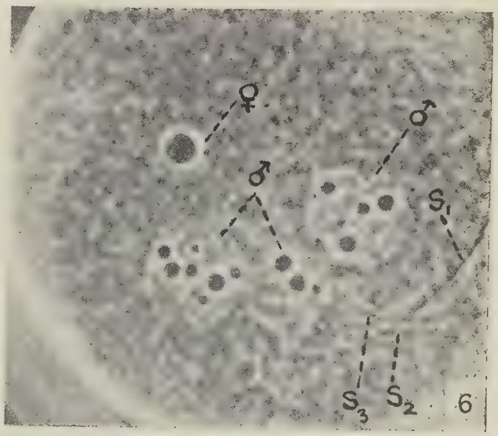

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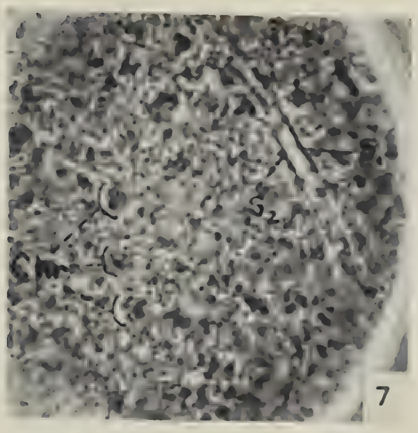
POLYSPERMY IN THE RAT AND RABBIT



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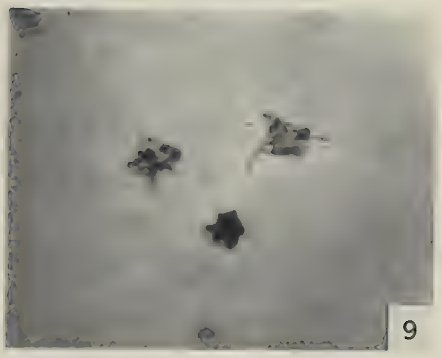

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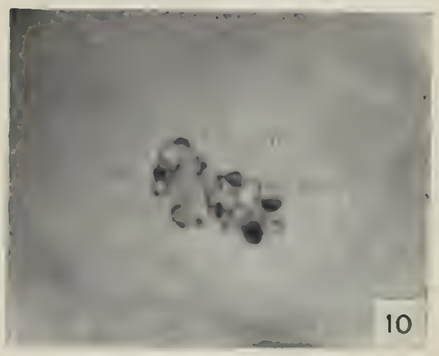

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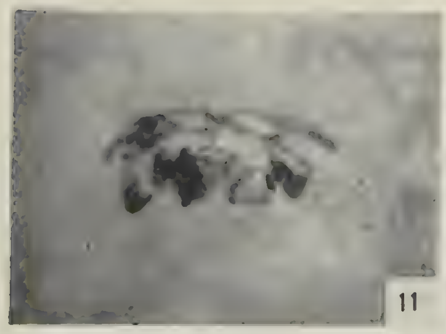

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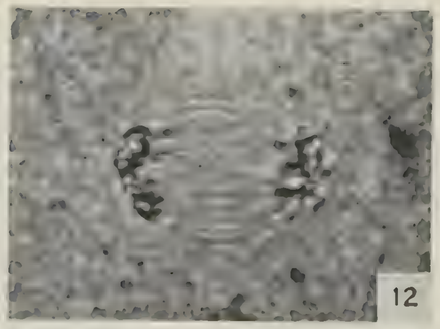

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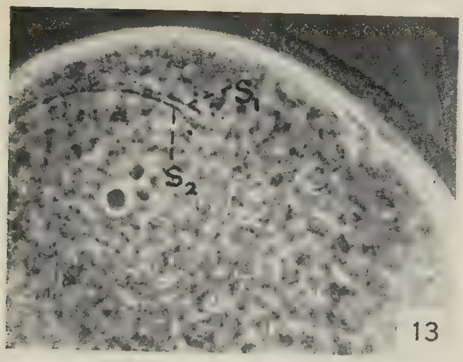
POLYSPERMY IN THE RAT AND RABBIT



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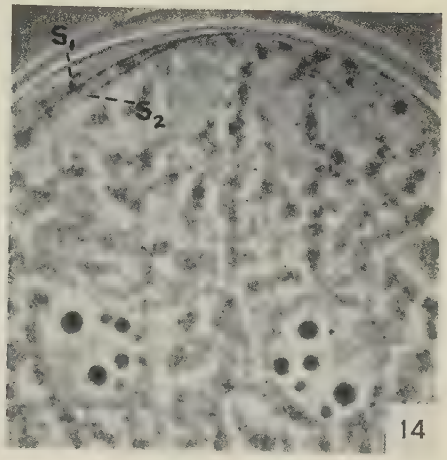

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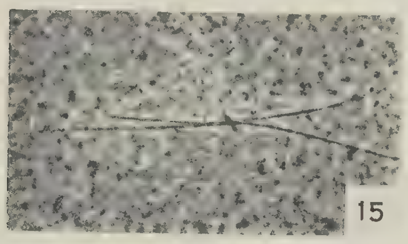

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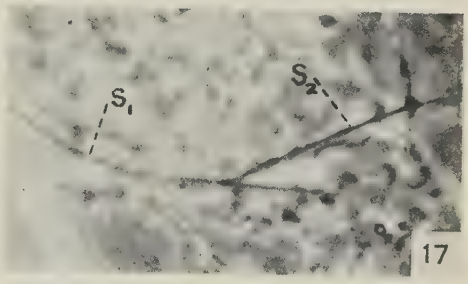

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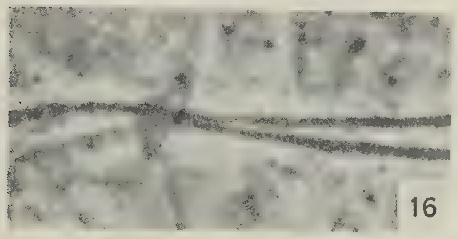

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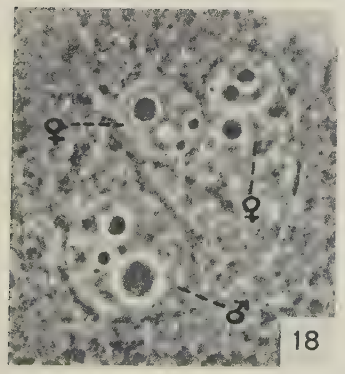

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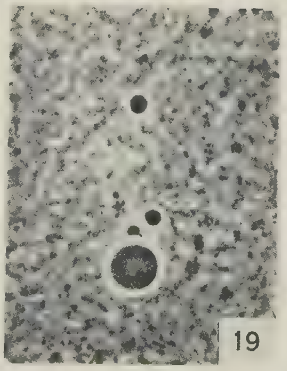

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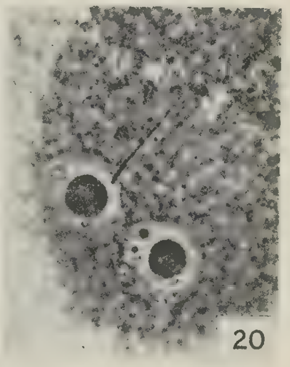

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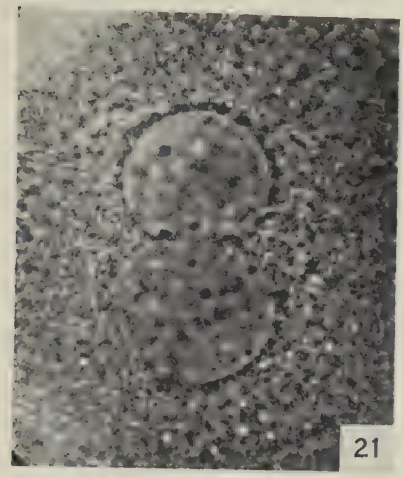
POLYSPERMY IN THE RAT AND RABBIT



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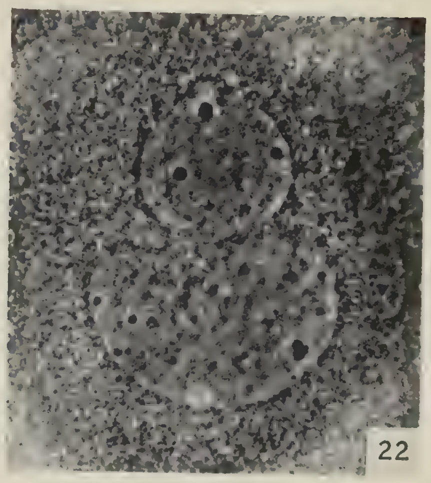

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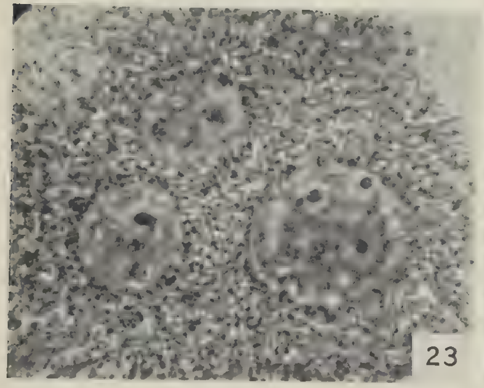

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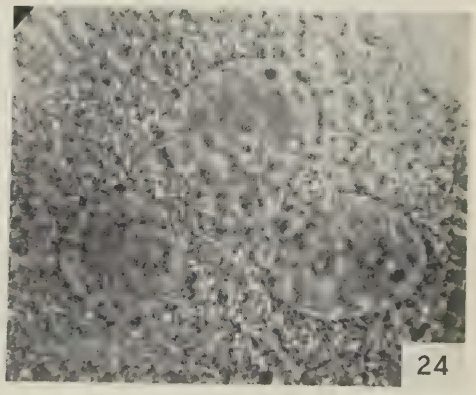

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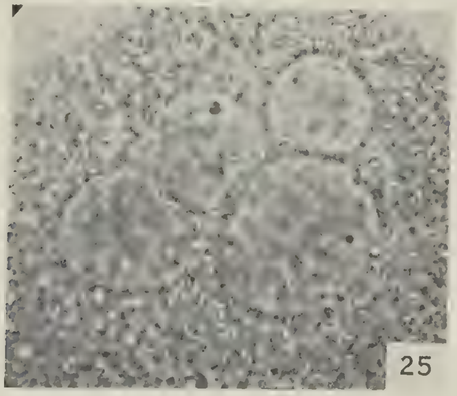

25

DISTRIBUTION OF SPERMS IN THE GENITAL TRACT OF THE FEMALE RABBIT AFTER COITUS

By A. W. H. Braden*

[*Manuscript received June* 16, 1953]

Summary

At each of the intervals, 1, 2, 3, 4, 6, 10, 13, 16, 20, and 28 hr after coitus, six rabbits were killed to provide data on sperm distribution.

The mean number of sperms in the cranial half of the vagina at 1 hr was 8.5×10^6. Between 1 and 4 hr it decreased to 1.9×10^6, after which there was no significant change.

The mean number of sperms in the caudal halves of the uterine horns was 0.9×10^6 at 1 hr and did not alter significantly up to 28 hr. The mean number of sperms in the cranial halves of the uterine horns was 0.05×10^6 1 hr after mating, and rose to 0.42×10^6 at 4 hr, after which there was no significant change.

Sperms were not found in the fallopian tubes until 3 hr after mating. The mean number in the entire tubes rose to about 5000 at 6 hr, after which it remained relatively constant. The mean number of sperms in the ovarian third of the tubes rose from 0 at 3 hr to 38 at 4 hr, 249 at 10 hr, and 478 at 28 hr. The increase from 3 to 10 hr was significant but that from 10 to 28 hr was not. In some animals, killed at 10-28 hr, the tubes were divided into thirds and it was found that 57 per cent. of the total number of sperms in the entire tubes were present in the uterine third, 35 per cent. were in the middle third, and 8 per cent. were in the ovarian third.

From this evidence, it is concluded that in rabbits the utero-tubal junction and the cervix are the most important barriers in limiting the passage of excessive numbers of sperms to the site of fertilization.

Above a certain level, increase in the number of sperms deposited in the vagina at coitus apparently has no effect on the total number of sperms entering the fallopian tubes.

The location and state of the eggs and their membranes were noted in the rabbits killed 10-28 hr after coitus. The eggs were mainly found in the middle third of the tubes, indicating rapid passage through the upper third. Ovulation had begun in all animals by 10 hr after mating, but was apparently not complete. Nevertheless, five out of eight eggs examined by histological methods at that time had been penetrated by sperms. The site of fertilization in the rabbit probably includes the whole ampulla, which constitutes about one-half of the fallopian tube.

I. Introduction

It has long been known that although very large numbers of sperms are normally deposited in the vagina or uterus at coitus in mammals, very few

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subsequently attain the site of fertilization (Tafani 1889; Sobotta 1895; Sobotta and Burckhard 1910; Hammond 1925). More recently, counts have been made of the actual number of sperms present at this location after mating. Hammond and Walton (1934) recovered 18, 27, 75, and 1600 sperms from the periovarial bursae of four ferrets, and Blandau and Money (1944), Austin (1948), and Blandau and Odor (1949) found 5-100 sperms in the rat ampulla. The ampulla is thought to be the site of fertilization in most mammalian species. For the entire fallopian tubes, numbers of the order of 2000-8000 sperms have been recorded in the ewe (Warbritton *et al.* 1937) and the rabbit (Austin 1948; Chang 1951*a*, 1951*b*).

The hypothesis that an important and overt function of the female genital tract is to prevent excessive numbers of sperms gaining access to the eggs during their penetrable life has been put forward recently (Austin and Braden 1952). The investigations to be reported in the present paper supply information on the control of sperm numbers in the rabbit. Studies on the distribution of sperms in the female genital tract after coitus have previously been reported for two other species. In sheep, Warbritton *et al.* (1937) found that the mean number of sperms in the vagina, uterus, and tubes of 20 ewes, killed 2 and 11 hr after mating, was 920,000, 76,000, and 6500 respectively. In the rat, in which intra-uterine insemination apparently occurs (Hartman and Ball 1930), the mean number of sperms recovered from the uterine horns was 58×10^6, whereas mean values as low as 860 and 24 sperms respectively were recorded for the isthmus and ampulla of the tubes (Blandau and Odor 1949). The present study shows that in the rabbit the utero-tubal junction, the cervix, the uterine horns, and the isthmus of the tubes, all operate, though with varying degrees of effectiveness, in limiting the number of sperms which reach the ampulla. The most important of these structures in the rabbit are the utero-tubal junction and the cervix, whereas in the rat the utero-tubal junction and the isthmus are entirely responsible for the enormous reduction in sperm number between the uterus and ampulla. In the ewe, on the other hand, it appears that the cervix plays the major part in limiting the numbers of sperms passing up the tract.

Information on the rate of passage of sperms through the female genital tract in the rabbit has also been gained. Heape's (1905) observation that sperms take about 4 hr to reach the ovarian end of the fallopian tube in the rabbit is confirmed, and it is shown that the numbers of sperms present here increases considerably in subsequent hours.

II. METHODS

Seventy-five virgin female rabbits, 4-12 months of age, and principally of the albino type, were used. Each was allowed to copulate once or twice with vigorous males. The does were killed at 1, 2, 3, 4, 6, 10, 13, 16, 20, and 28 hr after coitus and their genital tracts removed. Each tract was divided into nine parts: the upper or cranial half of the vagina, the upper and lower halves of each uterine horn, and the upper, or ovarian, third and the lower, or uterine, two-thirds of each fallopian tube. In some instances each tube was divided into upper, middle, and lower thirds. The caudal half of the vagina was not examined. The portions of uterus and vagina were flushed separately with 3.5 ml distilled water and the total sperm content of each washing was estimated by means of a haemocytometer. The sections of fallopian tube were each flushed with approximately 1 ml normal saline solution, and the washings were centrifuged at about 2,500 r.p.m. for 10 min. This procedure was employed to concentrate the sperm suspension as the number of sperms in the tube is relatively small. The bulk of the supernatant fluid was drawn off and the remainder was agitated vigorously with a fine pipette. An aliquot of the centrifuged suspension was placed on a slide, a coverslip with greased edges placed over it, and the total number of sperms present was counted.

The efficiency of the method of concentrating the tubal washings was low unless a drop of wetting agent ("Teepol," diluted 1 in 5000) was added to the washings before centrifugation. The use of centrifuge tubes treated with silicone was also found to be advantageous. With these modifications, the recovery, when a known number of sperms was used, was consistently between 40 and 60 per cent. A correction factor of 2 was therefore applied to the estimates of sperm number in the tubes.

III. Results

The mean numbers of sperms in the various segments of the female genital tract 1-28 hr after coitus, together with the range of values, are shown in Table 1, and the changes in the distribution of sperms with time are illustrated in Figure 1. The mean value at each interval is based on six rabbits.

The mean number of sperms found in the upper half of the vagina 1 hr after mating was 8.52×10^6. In subsequent hours the number decreased from 7.83×10^6 at 2 hr to 1.86×10^6 at 4 hr, after which no significant change took place up to 28 hr. The mean value for the number of sperms in the lower or caudal halves of the uterine horns in each rabbit 1 hr after coitus was 0.93×10^6; estimates for subsequent intervals did not vary significantly from this figure. The number of sperms recovered from the upper or cranial halves of the uterine horns 1 hr after mating was 0.052×10^6. The number at 2 hr was similar but between 2 and 4 hr it rose to 0.42×10^6, after which there was no significant change up to 28 hr.

Sperms were not found in the fallopian tubes until 3 hr after mating, when the mean number present was 56 per animal. These sperms were confined to the lower or uterine segment of the tubes. The mean number of sperms in the entire tubes reached a level of 5158 at 6 hr, after which it remained relatively constant up to 28 hr. In the upper or ovarian third of the tubes, the mean number of sperms increased from 0 at 3 hr to 38 at 4 hr, 84 at 6 hr, and 249 at 10 hr, which is the approximate time of ovulation in the rabbit. From 10 to 28 hr a further rise in the mean sperm number to 478 occurred, but was not significant, owing to the high variation between animals (Table 1). On the other hand, the increase in sperm number from 3 to 10 hr was significant.

TABLE 1

SPERMS RECOVERED FROM THE GENITAL TRACT OF THE RABBIT 1-28 HR AFTER COITUS

Interval Between Coitus and Slaughter (hr)	Vagina, Upper Half (millions)	Uterus		Fallopian Tubes	
		Lower Half (millions)	Upper Half (millions)	Total	Ovarian Third
1	8·52 (1·96-12·2)*	0·93 (0·47-1·79)*	0·052 (0·01-0·10)*	—	—
2	7·83 (4·0-17·1)	1·16 (0·66-1·64)	0·042 (0·01·0·09)	—	—
3	6·03 (0·20-15·0)	1·15 (0·56-1·55)	0·13 (0·04-0·28)	55·7 (0-180)*	—
4	1·86 (0·25-3·1)	0·84 (0·30-1·30)	0·42 (0·10-0·68)	1775 (474-3516)	38 (0-88)*
6	1·41 (0·26-3·5)	1·26 (0·22-2·65)	0·38 (0·12-1·10)	5158 (698-12,560)	84 (0-180)
10	0·64 (0·20-1·03)	1·33 (0·19-3·59)	0·55 (0·09-1·20)	3925 (2000-7608)	249 (96-720)
13	2·76 (0·04-4·48)	1·51 (0·04-2·72)	0·39 (0·01-0·89)	4479 (112-7324)	208 (15-480)
16	0·58 (0·08-1·64)	1·24 (0·37-2·71)	0·41 (0·10-0·71)	3566 (1976-6986)	291 (20-546)
20	2·11 (0·32-6·0)	0·87 (0·11-2·03)	0·31 (0·05-0·62)	4717 (662-8194)	343 (90-736)
28	1·75 (0·36-5·0)	1·18 (0·58-1·86)	0·55 (0·13-1·36)	4353 (1860-7742)	478 (100-956)

* The mean value and the range of values from six animals.

In five animals figures ranging from 20,000 to 50,000 were obtained as estimates of the total number of sperms present in the tubes. These values were exceptionally high and were excluded when calculating the mean values recorded in the foregoing paragraphs. In two of the five rabbits, the number of sperms recovered from one tube was within the normal range, whereas the number recovered from the other tube was about 10 times larger.

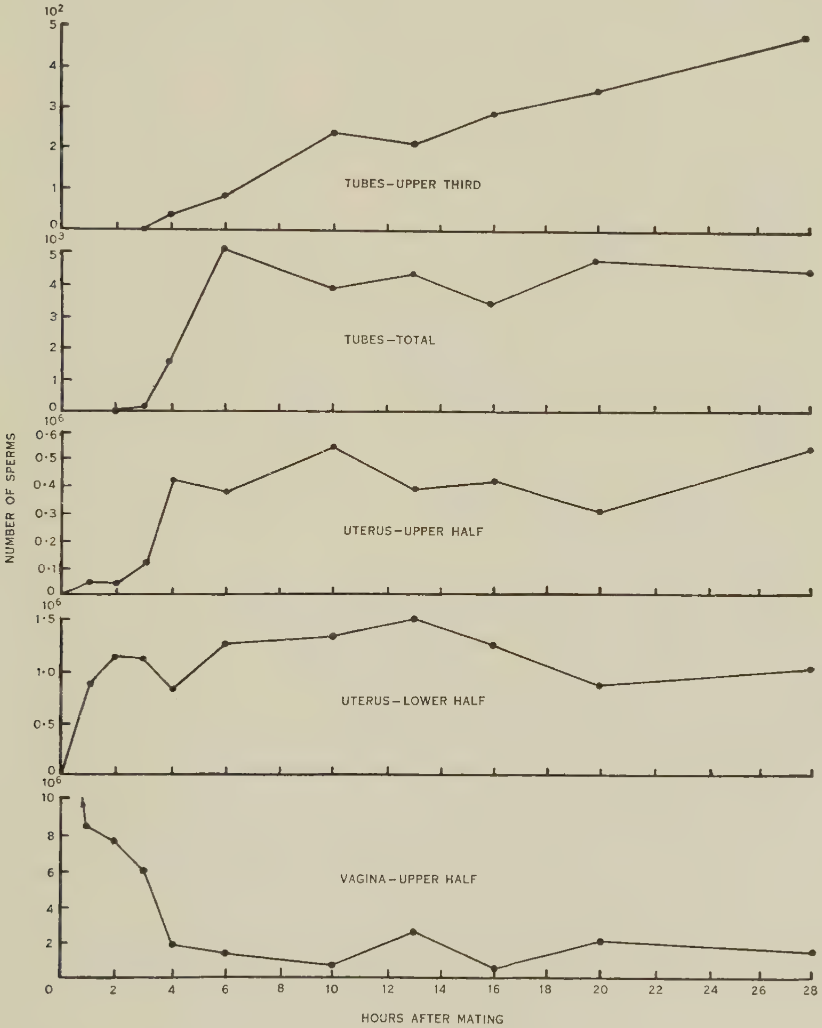

Fig. 1.—Changes in the number of sperms in various sections of the genital tract relative to time.

In some animals, the tubes were separated into ovarian, central, and uterine thirds, and separate sperm counts were made on each portion. The results from these animals are summarized in Table 2. The decrease in the mean number of sperms in the uterine third, from 4481 at 10 hr to 1806 at 28 hr, is not significant, nor is the increase in the mean number in the ovarian third from 183 at 10 hr to 478 at 28 hr. The results for the period 10-28 hr have therefore been combined to give overall means, which are also included in Table 2. In these animals the mean for the whole tubes was 4620 sperms, of which 2648, or 58 per cent., were in the uterine third, 1614, or 35 per cent., in the middle third, and 358, or 8 per cent., were in the ovarian third. The uterine third of the fallopian tube in the rabbit consists entirely of isthmus, the middle third partly of isthmus and partly of ampulla, and the ovarian third entirely of ampulla.

TABLE 2

SPERMS RECOVERED FROM THE FALLOPIAN TUBES 10-28 HR AFTER COITUS

Interval Between Coitus and Slaughter (hr)	No. of Rabbits	Fallopian Tubes		
		Uterine Third	Middle Third	Ovarian Third
10	2	4481 (2198-6764)*	1580 (748-2412)*	183 (96-270)*
13	2	4757 (4012-5502)	575 (322-828)	246 (118-374)
16	5	1854 (1054-4394)	1590 (708-2652)	345 (214-546)
20	6	2836 (170-5372)	1539 (402-3664)	343 (90-736)
28	6	1806 (660-3548)	2068 (976-4044)	478 (100-956)
10-28	21	2648	1614	358

* The mean value and range of values.

Note was taken of the location of the eggs in rabbits killed 10-28 hr after mating, and some of the eggs were fixed, embedded, and sectioned by methods previously described (Braden 1952) to determine whether they had been penetrated by sperms. Twenty-nine eggs were recovered from six rabbits killed 10 hr after coitus, and of these, 10 were found in the ovarian third and 19 in the middle third or, in one animal, the lower two-thirds of the tubes. Eight eggs were examined histologically and five of these had been penetrated by sperms. In one rabbit, two unpenetrated eggs were recovered from the lower two-thirds of the tubes. Five rabbits killed 13 hr after mating yielded 37 eggs, all of which were found in the middle third or the lower two-thirds of the tubes. Only one of the eggs was still embedded in the cumulus mass, four were completely denuded, and the rest had coronae of different degrees of thickness. At 16 hr 60 eggs were recovered from eight rabbits; 56 eggs were found in the middle third, and four eggs in the uterine third of the tubes. A thin layer of mucoprotein (the so-called "albumen" layer) was noticed around all except nine of these eggs when examined in the fresh state. Twenty-four of the eggs were examined histologically and all were found to be penetrated. A total of 51 eggs was recovered from six rabbits killed at 20 hr; nine were in the lower third and 42 in the middle third of the tubes. All had a layer of mucoprotein as thick as, or thicker than, the zona pellucida, but none had reached the 2-cell stage. Seven rabbits killed at 28 hr yielded 51 eggs, of which 15 were from the uterine third and 36 from the middle third of the tubes. One of the eggs was undivided, nine were in the 2-cell stage, 37 in the 4-cell, three in the 6-cell, and one in the 8-cell stage.

In order to determine whether an elevation of the number of sperms in the tubes above normal would occur if the number in the uterus was considerably increased, the following test was made: Six rabbits were allowed to copulate with 4-10 males within 10-20 min. The does were killed 16-20 hr later and the sperm numbers counted. The mean number of sperms in the upper half of the vagina was found to be 7.31×10^6, which was about five times larger than the number present when the animals were mated once or twice only (Table 3). The mean number of sperms in the lower half of the uterus in animals mated 4-10 times was 2.83×10^6 as compared with 1.05×10^6 for animals mated once or twice only. In only three of the animals was the value outside the range previously obtained for animals mated once or twice and killed 16-20 hr later ($0.11\text{-}2.71 \times 10^6$). The mean number of sperms in the upper half of the uterus from animals mated 4-10 times was 0.59×10^6, which is little different from the value obtained from animals mated once or twice only (0.36×10^6). In only two of the animals mated 4-10 times was the sperm number in the upper half of the uterus above the normal range ($0.05\text{-}0.71 \times 10^6$). Sperm counts were made on the tubes of one of these two animals and on the tubes of two other animals, one of which had 4.35×10^6 in the lower half of the uterus, though only 0.52×10^6 in the upper half. In each instance, the estimate for the total number of sperms in the tubes was less than the mean value of 4142 obtained from animals mated once or twice only. The actual values were 2272, 2640, and 3846, which were well within the normal range (662-8194). The mean number of sperms in the upper third of the tubes after multiple copulation in the three animals was 239, as compared with 317 for animals which copulated once or twice.

The correlation between the number (x) of sperms in the various segments of the genital tract was examined for 41 animals killed 10-28 hr after coitus. In place of the actual numbers the quantities $\log(x + 1)$ were considered as these follow approximately a normal distribution. There was a highly significant correlation ($r = 0.642$, $n = 82$, giving $P < 0.001$) between the transformed numbers of sperms in the upper and lower halves of the uterine horns. However, no significant correlation could be demonstrated between the transformed numbers of sperms in the upper half of the vagina and the corresponding value for the lower half of the uterus. Likewise, the number of sperms in a uterine horn appeared to have no relation to the number in the corresponding tube (Fig. 2).

IV. Discussion

The investigations reported in this paper lay further emphasis on the fact that, in mammals, only a very small proportion of the sperms deposited in the female tract at coitus subsequently reach the neighbourhood of the eggs. In the rabbit, only one sperm out of every 10,000-100,000 sperms ejaculated attains the ampulla of the fallopian tube, which is the site of fertilization. In the ewe

TABLE 3

DISTRIBUTION OF SPERMS IN RABBITS MATED 4-10 TIMES AND KILLED 16-20 HR LATER COMPARED WITH THOSE MATED ONCE OR TWICE ONLY

No. of Copulations	No. of Rabbits	Vagina, Upper Half (millions)	Uterus		Fallopian Tubes	
			Lower Half (millions)	Upper Half (millions)	Total	Ovarian Third
1-2	12*	1·50 (0·08-6·0)†	1·05 (0·11-2·71)†	0·36 (0·05-0·71)†	4142 (662-8194)†	317 (20-736)†
4-10	6‡	7·31 (3·55-16·7)	2·83 (1·43-4·35)	0·59 (0·11-1·28)	2919 (2272-3846)	239 (192-326)

* These figures are taken from Table 1 (16-hr and 20-hr intervals).

† The mean value and range of values.

‡ The figures for the tubes in this group are based on only three of these six animals.

and the rat, the proportion is even smaller: only about one sperm out of every 1-3 × 10^6 reaches the site of fertilization (Warbritton *et al.* 1937; Blandau and Odor 1949).

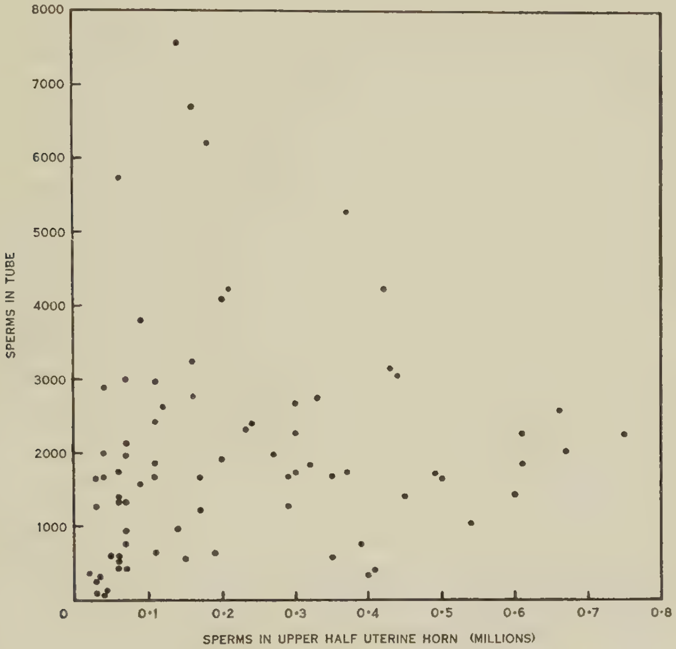

Fig. 2.—Lack of correlation between the number of sperms in the upper half of a uterine horn and the number in the corresponding fallopian tube. The results were obtained from animals killed 6-28 hr after coitus.

Of importance in limiting the passage of sperms up the tract are the cervix, the uterus, the utero-tubal junction, and the isthmus of the tubes. The relative importance of the various structures differs between species. In the rat, the utero-tubal junction plays the major role in restricting the passage of sperms to the ampulla, whereas the isthmus is of less importance. The cervix and uterine horns apparently have no restrictive action. Available evidence indicates that, in the ewe, the cervix is by far the most important structure in this respect; the utero-tubal junction and isthmus are not nearly as effective in restricting sperm passage (Warbritton *et al.* 1937; Braden, unpublished data). In the rabbit the utero-tubal junction and the cervix limit sperm passage most, whereas the isthmus and the uterine horns have only a slight effect. The relative effectiveness of the various parts in the rabbit may be stated as follows: The utero-tubal junction allows only one sperm of every 160 present in the upper half of the uterus to enter the tubes, and only one of every 40 sperms ejaculated into the vagina traverses the cervix. Approximately one-quarter of the number of sperms entering the tubes passes through the isthmus to the ampulla, and about one-third of the number entering the uterus reaches the upper half of that organ.

The ability of the cervix to restrict the passage of sperms into the uterus in the rabbit has been demonstrated by the results obtained in the present study. The number of sperms deposited in the vagina by a normal male rabbit is about 60×10^6, though this value is subject to considerable variation (Asdell 1946). In comparison the number of sperms recovered from the upper half of the vagina 1 hr after mating was only 8.5×10^6. Evidently, the great majority of the sperms originally deposited in the vagina by the male are quickly lost to the exterior. Furthermore, the mean number in the remainder of the genital tract was about 1.5×10^6 which is considerably less than one-fortieth of the number originally introduced into the vagina. When the number of sperms deposited in the vagina was increased about five times by multiple copulation, the number of sperms found in the rest of the genital tract was increased only 2.5 times, a further indication of the restrictive action of the cervix.

The slight reduction in number which occurs as the sperms pass up the uterus may be imputed simply to the dissemination of the sperms over the relatively large endometrial surface. The strong positive correlation between the numbers of sperms in the upper and lower halves of the uterine horns lends support to this hypothesis. No such simple relation exists, however, between the number of sperms in the upper half of the uterus and the number in the lower segment of the tubes, suggesting that some other mechanism is in operation at the junction between the uterus and the tubes. A mean number of 0.42×10^6 sperms was recovered from the upper half of the uterus compared with 2650 from the lower third of the tubes (Table 2): a ratio of about 160:1. In contrast, the number of sperms in the uterus of the rat is about 30,000 times the number in the tubes (Blandau and Odor 1949), and in the ewe the ratio of the number of sperms in the upper part of the uterus to the number in the lower third of the tubes is about 10:1 (Braden, unpublished data).

Morphological studies in a number of species of the region where the uterus and the fallopian tubes are confluent have shown that the utero-tubal junction is well adapted to restrict the passage of sperms (Kelly 1927; Anderson 1928; Lee 1928). The structure has a well-developed circular layer of smooth muscle and numerous polyp-like folds of the mucosa, the latter making the lumen narrow and tortuous. The mucosal folds guarding the tubal ostium apparently act as valves if intra-uterine pressure is elevated.

In five rabbits, the number of sperms recovered from the tubes was exceptionally high—20,000-50,000. This may have been the result of contamination with uterine washings in the recovery technique, though care was taken to avoid such an occurrence. On the other hand, it may be that the utero-tubal junction was not functioning normally in these animals. In two of the animals the sperm number in one tube was about 2500, which is well within the normal range, whereas the number in the other tube was 25,000-50,000.

Some further reduction in sperm number is evidently effected by the isthmus of the fallopian tube in both the rabbit and the rat. In the rat, a mean number of 860 sperms were recovered from the isthmus and only 24 from the ampulla

(Blandau and Odor 1949). The corresponding values for the rabbit found in the present study were about 3500 and 1000. The reduction in sperm number accomplished by the isthmus in the rabbit is not nearly as great as in the rat, and is even less if the number of sperms which actually penetrate the zona of the eggs is also taken into account. The mean number of eggs per animal recovered from rabbits killed 16-20 hr after coitus in the present series was 7.9 and the mean number of sperms which had penetrated them about 490 per rabbit. Corresponding values obtained for the rat were 9.2 eggs and 12 penetrating sperms per animal (Braden, unpublished data). When these sperms are added to the number in the ampulla the ratio of the mean number of sperms in the isthmus to that in the ampulla becomes 24:1 for the rat and 2.3:1 for the rabbit. It must be remembered, however, that these estimates of sperm number in the fallopian tubes are only very approximate, as the technique employed for their recovery is relatively crude.

The present experiments have indicated that the mean number of sperms in the tubes of the rabbit 6-28 hr after coitus probably lies between 4000 and 5000 and that, above a certain level, increase in the number of sperms inseminated has no significant effect on this value. These findings are thus in agreement with those of Austin (1948) and Chang (1951*b*). Austin estimated that 2000-4000 sperms were present in individual tubes when rabbits were artificially inseminated with 100 × 10^6 sperms. When less than 1 × 10^6 sperms were introduced into the vagina, the number in the tubes was much less. Chang obtained a mean value of 5120 for the number of sperms present in the tubes of five mated rabbits, and 6480 for the tubes of six animals artificially inseminated with 20 × 10^6 sperms. When only 0.6-1.8 × 10^6 sperms were inseminated, the mean number recovered from the tubes of five rabbits was 88. Chang concluded that the number of sperms present in the tubes was relatively constant if 10 × 10^6 or more sperms were inseminated. He postulated that the uterine cervix and the isthmus of the fallopian tube functioned as barriers against the passage of large numbers of sperms. It is worthy of note that the number of sperms in the tubes is essentially the same whether artificial insemination or natural mating is employed. The available evidence, therefore, supports the hypothesis that an important function of the female genital tract is to limit the number of sperms passing to the site of fertilization. This limitation is presumably necessary because the presence of excessive numbers of sperms about the eggs at fertilization may well increase the danger of polyspermy. Furthermore, it is quite possible that even in monospermic eggs very large numbers of supplementary sperms in the perivitelline space would interfere with its normal development.

From the present results it can be seen that passage of the sperms through the cervix of the rabbit is undoubtedly rapid, for maximal numbers were present in the lower half of the uterus within 1 hr after coitus. A few sperms reached the upper half of the uterus within 1 hr, but maximal numbers were not found until 4 hr after mating. There is some difference of opinion on the mode of transport of the sperms through the rabbit uterus: some workers have considered that the sperms ascend by means of their own motility (Marshall 1922; Parker 1931; Chang and Pincus 1951), whereas others contend that the motility of the uterus is largely responsible for sperm passage (Krehbiel and Carstens 1939; Schilling and Kordts 1952). The present results suggest that both mechanisms may operate; that the sperms which reach the upper half of the uterus during the first hour are transported by the activity of the tract, and that those which arrive at the same location 2-3 hr later do so mainly by their own motility. The findings are in accord with those of Florey and Walton (1932), who reported that increasing numbers of sperms were found near the opening of a uterine fistula 20 min to 2 hr after copulation in the rabbit.

Sperms were not recovered from the tubes until 3 hr after mating, and at this time they were confined to the lower part of the tubes. The sperms began to accumulate in the ovarian third of the tubes at about 4 hr. This confirms Heape's (1905) observation that sperms took 4 hr to reach the infundibulum in the rabbit. Agreement is also found with San Martin (1951), who reported solitary spermatozoa in the upper reaches of rabbit tubes 4 hr after coitus and somewhat larger numbers at this site at 6 hr. Other workers, however, have reported shorter intervals. Hensen (1876) found sperms at the infundibulum 2¾ hr after coitus in one animal, and Parker (1931) recorded finding them there in two rabbits at 3 hr, whereas Chang (1952) claims to have recovered sperms from the upper part of the fallopian tube as early as 1 hr after mating.

Reference has been made to the site of fertilization in mammals, but its location is somewhat ill-defined. Anderson (1927) considered that it was restricted to the upper third of the tube, but the present results suggest that, in the rabbit at least, it probably includes the whole of the ampulla. This does not necessarily mean, however, that fertilization takes place over a considerable interval of time under normal circumstances, for the passage of the eggs through the upper third of the tubes is rapid, as has been shown in the present series, and by Pinner (1880), Assheton (1894), and Chang (1951*a*). Ovulation was evidently still proceeding in the rabbits killed 10 hr after mating, for the mean number of eggs recovered was 4.8 as compared with 7.7 eggs per rabbit from animals killed at 13-28 hr. Nevertheless, more than half of the eggs recovered at 10 hr were in the middle third of the tubes and five out of eight eggs examined had been penetrated by sperms. Penetration of the zona pellucida by supplementary sperms apparently continues up to about 6 hr after ovulation. About this time a layer of mucoprotein begins to accumulate around the zona, and this probably renders the eggs impenetrable. Eggs were found mainly in the middle third of the tubes at this time, which indicates that penetration of the zona does not normally occur in the lower third of the tubes.

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METABOLIC STUDIES OF WASHED AND DILUTED RAM AND BULL SPERMATOZOA

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Summary

The total oxygen uptake of ram and bull spermatozoa in a 3-hr period at 37°C was not affected by washing four times in a sodium phosphate-fructose diluent nor by centrifuging four times without washing.

The total lactic acid production of the spermatozoa of both species was significantly reduced by washing four times but was unaffected by merely centrifuging. Washed ram and bull spermatozoa produced more lactic acid when 0.004M KCl was added to the sodium phosphate-fructose diluent; the glycolysis of ram spermatozoa washed in the potassium-containing diluent was almost equal to the unwashed control.

The total oxygen uptake of ram and bull spermatozoa was not affected by a tenfold dilution from an initial cell concentration of 200×10^6 cells/ml when the dilution was made in either a potassium-containing or a potassium-free diluent. Dilution significantly reduced the lactic acid production of the spermatozoa of both species, particularly when the dilution was made in a potassium-free diluent. The addition of 0.004M KCl to the sodium phosphate-fructose diluent increased the glycolysis of diluted ram spermatozoa but not that of diluted bull spermatozoa.

It is concluded from these experiments that repetitive washing and dilution are similar in their effect on the metabolism of ram and bull spermatozoa and that it is the glycolytic rather than the oxidative mechanisms that are damaged. Potassium appears to be one important component lost from spermatozoa during both treatments.

I. Introduction

In previous studies (White 1953*a*) it was found that ram, bull, and rabbit spermatozoa could be washed once in a sodium phosphate-fructose diluent with little or no impairment of motility or metabolism. On washing twice there was a decrease in motility, total oxygen uptake, and total lactic acid production over the 5-hr period of the experiments. While the effect of repeated washing on the total oxygen consumption was shown to be largely due to the removal of bacteria otherwise proliferating in the suspensions during the later hours, it was believed that the decreased lactic acid production was associated with the spermatozoa. To examine these conclusions more critically the effect of washing on the oxygen uptake and glycolysis of ram and bull spermatozoa has been investigated, over a 3-hr period to minimize the influence of bacteria, with the number of washings increased to four to accentuate this effect.

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Motility studies of ram and bull spermatozoa after washing four times have already been reported (White 1953*b*). In these experiments it was shown that the adverse effect of repeated washing on motility could be largely prevented by the inclusion of potassium in the diluent, and furthermore, that potassium had a beneficial action on spermatozoa at moderate dilutions. Similar results have been obtained by Blackshaw (1953*a*, 1953*b*). A survey of the available literature revealed only one reference to the effect of potassium on the metabolism of mammalian spermatozoa (Lardy and Phillips 1943), although numerous studies have been made of other cells and tissues, mostly in relation to the importance of potassium in carbohydrate metabolism. Thus it has been shown that potassium stimulates the aerobic metabolism of brain (Ashford and Dixon 1935; Lipsett and Crescitelli 1950), fermentation by yeast (Farmer and Jones 1942), and glycogen deposition in rat liver slices (Buchanan, Hastings, and Nesbett 1949*a*, 1949*b*). It was of interest therefore to study the effect of potassium on the metabolism of washed and diluted spermatozoa, and the results of these experiments are reported here.

II. MATERIALS AND METHODS

Bull semen was collected by the artificial vagina and ram semen by electrical stimulation (Gunn 1936). Only morphologically normal ejaculates of good motility were employed. Experiments with ram semen were started immediately after collection and those with bull semen within 2 hr. The semen was stored during this period at about 10°C, precautions being taken to avoid cold shock.

Suspensions were prepared by diluting 0.5-1.0 ml of semen 1 : 10 in a graduated centrifuge tube, and the spermatozoa were washed by spinning this suspension four times at 1500 r.p.m. (about 300g) for 10 min. The supernatant that was drawn off and replaced by diluent after each centrifuging and the spermatozoa redispersed. Centrifuged but unwashed spermatozoa suspensions were prepared by centrifuging the diluted semen four times and redispersing the cells after each spinning without removing the supernatant.

In the dilution experiments spermatozoal counts were made with the neat semen, using a haemocytometer under high power. The semen was then serially diluted to give cell concentrations of *c.* 200×10^6 and 20×10^6 cells/ml.

Each experiment was of 3 hr duration, the gas phase being air. The Warburg bath was set at 37°C with a shaking rate (114 strokes/min) sufficient to keep the liquid phase saturated with oxygen. Oxygen uptake was measured at hourly intervals by the direct Warburg technique (Umbreit, Burris, and Stauffer 1949).

Lactic acid was estimated on the flask contents by the method of Barker and Summerson (1941) at the start and end of experiments.

The isotonic diluents of pH 7.0 had the following composition:

Sodium: 0.032M $NaH_2PO_4.H_2O$, 0.048M $Na_2HPO_4.12H_2O$, 0.040M NaCl, 0.022M fructose.

Sodium-potassium: 0.032M $NaH_2PO_4.H_2O$, 0.048M $Na_2HPO_4.12H_2O$, 0.036M NaCl, 0.004M KCl, 0.022M fructose.

In the analysis of variance the total oxygen uptake and the total lactic acid production over the 3-hr period have been used as unit observations and the interaction mean square as the error term. The treatment sum of squares has been partitioned to give selected comparisons which are non-orthogonal.

Table 1

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE OXYGEN UPTAKE OF RAM SPERMATOZOA AT 37°C

Treatment	Ejaculate	$O_2/10^8$ Cells/Hr (μl)			Total Oxygen Uptake
		First Hr	Second Hr	Third Hr	
Unwashed	1	9·7	5·9	5·5	21·1
	2	12·7	8·4	8·6	29·7
	3	11·2	7·0	6·6	24·8
	Mean	11·2	7·1	6·9	25·2
Centrifuged but unwashed	1	8·7	5·7	6·9	21·3
	2	10·2	6·9	7·3	24·4
	3	10·6	5·5	5·8	21·9
	Mean	9·8	6·0	6·7	22·5
Washed	1	10·7	7·4	5·4	23·5
	2	11·2	9·7	5·6	26·5
	3	11·0	7·1	6·2	24·3
	Mean	11·0	8·1	5·7	24·7

III. Results

The effect of washing four times on the oxygen uptake and glycolysis of ram and bull spermatozoa over a 3-hr period was studied with three ejaculates from each species, using the sodium phosphate-fructose diluent. A centrifuged but unwashed group was included in these experiments in addition to the unwashed control, to assess the mechanical effect of centrifuging on the oxygen uptake of the spermatozoa. Tables 1 and 2 give the results for the oxygen uptake of ram and bull spermatozoa respectively. These data were subjected to analysis of variance and it is clear from the summary (Table 3) that there was no significant difference between either treatments or ejaculates in the two species.

The corresponding values for lactic acid production are set out in Table 4. These also were subjected to analysis of variance (Table 5) which showed that washing four times significantly reduced the amount of lactic acid produced by ram and bull spermatozoa. Lactic acid production by the centrifuged but unwashed spermatozoa of both species was not, on the other hand, significantly different from that in the control specimens that had been neither centrifuged nor washed. The ejaculates from the bulls used in this experiment varied significantly in their capacity to produce lactic acid; this was not so, however, with the ram ejaculates.

TABLE 2

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE OXYGEN UPTAKE OF BULL SPERMATOZOA AT 37°C

Treatment	Ejaculate	$O_2/10^8$ Cells/Hr (μl)			Total Oxygen Uptake
		First Hr	Second Hr	Third Hr	
Unwashed	1	6·5	5·0	5·5	17·0
	2	8·0	5·5	5·1	18·6
	3	—	—	—	32·0*
	Mean				22·5
Centrifuged but unwashed	1	7·3	5·8	6·0	25·1
	2	8·1	7·7	4·5	20·3
	3	14·5	12·1	9·2	35·8
	Mean	10·0	8·5	6·6	25·1
Washed	1	5·6	5·8	6·5	17·9
	2	8·3	10·2	3·7	22·2
	3	15·2	9·9	7·2	32·3
	Mean	9·7	8·6	5·8	24·1

* This value has been calculated by the method described in Cochran and Cox (1950) for estimating missing data.

TABLE 3

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE TOTAL OXYGEN UPTAKES IN TABLES 1 AND 2

Source of Variation	Ram		Bull	
	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
Between treatments:				
Unwashed *v.* washed	1	0·1	1	1·5
Unwashed *v.* centrifuged	1	4·1	1	3·7
Between ejaculates	2	7·2	2	7·9
Interaction (error)	4	258	3*	259

* The degrees of freedom have been reduced by one because of the missing value inserted in Table 2

It has been shown previously (White 1953*b*) that the addition of potassium to the sodium diluent almost completely restores the motility of repeatedly washed ram spermatozoa, and markedly increases that of washed bull spermatozoa. Experiments were therefore undertaken to see if potassium had a similar effect on the depressed glycolysis of the washed spermatozoa. Five ram and five bull ejaculates were used. With each ejaculate comparisons were made between spermatozoa washed four times in the potassium-containing and potassium-free diluents respectively, with an unwashed specimen in the potassium-free diluent as a control. The results are given in Table 6 and the summary of the analyses of variance in Table 7. It is clear that lactic acid production by the washed spermatozoa of both species is significantly greater in the presence of 0.004M KCl. The addition of potassium to the washed ram spermatozoa almost restored glycolysis to the level of the unwashed controls.

TABLE 4

RELATIVE EFFECTS OF WASHING AND CENTRIFUGING FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT ON THE GLYCOLYSIS OF RAM AND BULL SPERMATOZOA AT 37°C

Values represent μg lactic acid produced by 10^8 spermatozoa in 3 hr

Ejaculate	Unwashed		Centrifuged but Unwashed		Washed	
	Ram	Bull	Ram	Bull	Ram	Bull
1	114	168	106	196	8	101
2	137	326	147	382	−3	173
3	82	182	117	153	−22	79
Mean	111	225	123	244	−6	118

TABLE 5

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 4

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
Unwashed *v.* washed	1	37·6**	11·6*
Unwashed *v.* centrifuged	1	0·4	0·3
Between ejaculates	2	1·7	14·6*
Interaction (error)	4	542	1503

* *P*<0·05.

** *P*<0·01.

A feature of mammalian spermatozoa is that their motility is decreased by dilution (Salisbury *et al.* 1943; Emmens and Swyer 1948; Cheng, Casida, and Barrett 1949; White 1953*b*; Blackshaw 1953*b*) even if the dilution is made in an isotonic buffered medium containing a glycolysable sugar. The damage caused by dilution might be expected to be similar to that produced by repeated washing, since in both procedures the concentration of seminal plasma constituents is reduced and the loss of intracellular material from the spermatozoa presumably accelerated thereby.

TABLE 6

EFFECTS OF WASHING FOUR TIMES IN SODIUM AND SODIUM-POTASSIUM DILUENTS ON THE GLYCOLYSIS OF RAM AND BULL SPERMATOZOA AT 37°C

Values represent μg lactic acid produced by 10^8 spermatozoa in 3 hr

Ejaculate	*A.* Unwashed in Sodium Diluent		*B.* Washed in Sodium Diluent		*C.* Washed in Sodium-Potassium Diluent	
	Ram	Bull	Ram	Bull	Ram	Bull
1	246	277	45	223	164	275
2	275	146	222	29	411	44
3	221	363	97	88	211	219
4	330	290	131	153	289	260
5	433	411	163	116	345	245
Mean	301	297	132	122	284	209

TABLE 7

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 6

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	26·2**	24·8**
B v. C	1	21·2**	6·1*
Between ejaculates	4	5·8*	5·8*
Interaction (error)	8	2739	3115

* $P<0{\cdot}05$.
** $P<0{\cdot}01$.

It was of interest therefore to see if dilution had a similar effect to repeated washing on the metabolism of spermatozoa and to investigate the influence of potassium. Four ejaculates from each species were used. Table 8 gives the lactic acid production and Table 10 the oxygen uptake of ram and bull spermatozoa incubated for 3 hr at 37°C after 1 : 10 dilution in sodium and sodium-potassium diluents, the cells being suspended initially in the sodium diluent at a concentration of 200×10^6 ml. Ideally, it would have been desirable to work at higher dilutions since previous motility studies have shown that the dilution phenomenon is greatest at very low cell concentrations; the degree of dilution possible in metabolic experiments is, however, restricted by the design of the Warburg apparatus and the sensitivity of the techniques used. The analyses of variance for the respiration data are summarized in Table 11 and show that the oxygen uptake of ram and bull spermatozoa was not significantly affected by dilution in either the sodium or sodium-potassium diluents. On the other hand, analysis of variance of the glycolysis results (Table 9) shows that dilution in the sodium diluent significantly reduced the lactic acid production of the spermatozoa of both species. The glycolysis of ram spermatozoa on dilution was significantly greater in the presence of potassium, but this effect was not seen with bull spermatozoa. Significant between-ejaculate variation in oxygen consumption (Table 11) occurred with the rams used in this experiment, whilst the lactic acid production of different ejaculates differed significantly in both species (Table 9).

TABLE 8

LACTIC ACID (μg) PRODUCED BY 10^8 RAM AND BULL SPERMATOZOA INCUBATED FOR 3 HR AT 37°C AFTER DILUTION IN SODIUM AND SODIUM-POTASSIUM DILUENTS

Ejaculate	*A*. Sodium Diluent (200×10^6 cells/ml)		*B*. Sodium Diluent (20×10^6 cells/ml)		*C*. Sodium-Potassium Diluent (20×10^6 cells/ml)	
	Ram	Bull	Ram	Bull	Ram	Bull
1	240	435	94	206	177	202
2	549	250	178	62	260	125
3	505	208	154	54	325	208
4	267	675	35	276	214	275
Mean	390	392	115	150	269	203

TABLE 9

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 8

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	48·4**	15·3**
B v. C	1	15·1**	0·7
Between ejaculates	3	9·9	6·0*
Interaction (error)	6	3128	7679

* $P<0·05$.

** $P<0·01$.

IV. Discussion

In these experiments it is shown that washing ram and bull spermatozoa four times in a sodium phosphate-fructose diluent causes a fall in their lactic acid production (Table 4). These results confirm those previously reported by White (1953*a*) in which it was demonstrated that even after two washings the lactic acid production of ram, bull, and rabbit spermatozoa was significantly impaired. The effect was much more striking after the exhaustive washing employed in the experiments described here and, as they were conducted over a shorter period, the results are less likely to be complicated by the proliferation of bacteria. On the other hand, the total oxygen uptake of ram and bull spermatozoa (Tables 1 and 2) in these experiments was not affected by repetitive washing.

Table 10

OXYGEN (μl) TAKEN UP BY 10^8 RAM AND BULL SPERMATOZOA INCUBATED FOR 3 HR AT 37°C AFTER DILUTION IN SODIUM AND SODIUM-POTASSIUM DILUENTS

Ejaculate	*A*. Sodium Diluent (200 × 10^6 cells/ml)		*B*. Sodium Diluent (20 × 10^6 cells/ml)		*C*. Sodium-Potassium Diluent (20 × 10^6 cells/ml)	
	Ram	Bull	Ram	Bull	Ram	Bull
1	43·2	24·3	49·0	31·4	50·0	37·4
2	27·7	33·2	26·9	27·4	40·5	36·4
3	21·3	21·5	41·3	30·8	35·0	23·1
Mean	33·3	28·6	41·7	26·6	41·5	30·3

Table 11

SUMMARY OF THE ANALYSES OF VARIANCE FOR THE DATA IN TABLE 10

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Between treatments:			
A v. B	1	3·8	0·5
B v. C	1	3·6	3·2
Between ejaculates	3	5·0*	4·4
Interaction (error)	6	3763	2273

* $P<0·05$.

The adverse effect of repeated washing would seem therefore to involve damage to the glycolytic rather than to the oxidative processes of the spermatozoa. Furthermore, the decreased glycolysis must be associated with the leaching out of intracellular components from the spermatozoa rather than with the mechanical damage due to centrifuging, since the spermatozoa that were centrifuged without washing produced as much lactic acid as the unwashed controls (Table 4).

The motility observations of White (1953*b*) and Blackshaw (1953*b*) suggested that potassium was an important substance lost from spermatozoa on repeated washing, and the metabolic studies reported here support this suggestion.

The effect of dilution on the metabolism of ram and bull spermatozoa (Tables 8 and 10) was similar to that of repetitive washing. Potassium loss from the spermatozoa cannot be the only factor involved, however, since the lactic acid production of ram spermatozoa diluted in the potassium medium was never as high as that of the undiluted controls, whilst potassium had no significant effect on the glycolysis of diluted bull spermatozoa (Table 9).

The observation that the respiratory rate of ram and bull spermatozoa was unaffected by a tenfold dilution from an initial cell concentration of 200×10^6/ml is supported by several workers (Lardy and Phillips 1941; Lardy, Winchester, and Phillips 1945; Salisbury 1946). At much higher cell concentrations Winchester and McKenzie (1941) working with ram, and Salisbury (1946) with bull spermatozoa, record a depression of oxygen uptake. As has been pointed out, however, by Lardy, Winchester, and Phillips (1945) this might well be the result of limited oxygen diffusion into the viscous liquid phase.

Mann and Lutwak-Mann (1948) found a similar fall (Table 8) in the lactic acid production of ram spermatozoa after a fivefold dilution from an initial concentration of 3×10^8 cells/ml. However, these workers also recorded a reduction of oxygen uptake on dilution. Whilst this might be so with some ejaculates, the results of the experiments reported here indicate that impairment of glycolysis precedes that of oxidative processes. Salisbury (1946) seems to be the only other worker who has studied the effect of dilution on the glycolysis of mammalian spermatozoa and his results appear to be at variance with those reported here, in that he was unable to detect any difference in the glycolysis of bull spermatozoa at dilutions of 1 : 4 and 1 : 16. His experiments were, however, conducted over a 10-day period and the results might easily have been complicated by bacterial growth. Furthermore, over this long period, the metabolism of the spermatozoa at the higher concentration may have been depressed by a fall in the pH of the diluent.

Apparently the only reference in the literature to the effect of potassium on the metabolism of mammalian spermatozoa is that of Lardy and Phillips (1943), who present evidence for the stimulatory action of potassium on both the respiration and glycolysis of bull spermatozoa washed twice in potassium-free Ringer phosphate. Whilst neither effect could be judged statistically significant, it is clear from their limited data that the effect of potassium on glycolysis is much greater than on respiration.

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STUDIES ON THE ALKALI METAL REQUIREMENTS OF RAM AND BULL SPERMATOZOA

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Summary

Rubidium was as effective as potassium in maintaining the motility of ram and bull spermatozoa washed four times in a sodium phosphate-fructose diluent. Caesium was less active, ammonium ions were ineffective, and lithium was toxic.

The motility of unwashed ram and bull spermatozoa was better maintained in a diluent with sole cation sodium than with potassium. However, the addition of sodium ions did not significantly increase the motility of ram and bull spermatozoa washed four times in the potassium diluent.

Neither 10^{-2}M acetylcholine nor 10^{-5}M physostigmine, singly or in combination, significantly modified the motility of ram and bull spermatozoa washed in the sodium diluent.

Desoxycorticosterone acetate (1 μg/ml) depressed the motility of ram spermatozoa washed in both potassium-free and potassium-containing diluents, but had no significant effect on bull spermatozoa.

I. INTRODUCTION

In previous studies it was found that the motility and glycolysis of ram and bull spermatozoa were depressed after repeated washing in a sodium phosphate-fructose diluent (White 1953*a*, 1953*b*). Potassium loss from the spermatozoa was apparently important, as the addition of 0.004M KCl to the diluent almost completely restored the motility and glycolysis of washed ram spermatozoa and increased that of washed bull spermatozoa. Lardy and Phillips (1943), working with bull spermatozoa, have noted a similar stimulation of glycolysis by potassium and the effect on motility has been corroborated for the spermatozoa of both species by Blackshaw (1953*a*, 1953*b*).

Lithium has been shown to depress the motility and glycolysis of human spermatozoa (Macleod, Swan, and Aitken 1949). No studies seem to have been made on the effect of rubidium or caesium on spermatozoa. The ability of rubidium to replace potassium, however, is apparently a general biological phenomenon (e.g. Macleod and Snell 1948; Gallego and Lorente de Nó 1947; Pressman and Lardy 1952).

Ludwig, Greig, and Peterson (1951) have studied the loss of potassium from human erythrocytes suspended in isotonic sodium bicarbonate and claim that it is reduced by acetylcholine and increased by physostigmine, suggesting that intracellular potassium levels are dependent upon cholinesterase activity. If a similar mechanism operates in mammalian spermatozoa the addition of

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acetylcholine and physostigmine might be expected to modify the motility of spermatozoa washed in a potassium-free diluent.

Desoxycorticosterone has a marked effect on tissue potassium *in vivo* (Overman 1951). Little is known about its action at the cellular level; it would seem, however, to modify the permeability of cells, particularly those of the renal tubules. Desoxycorticosterone might therefore be expected also to influence the response of spermatozoa to repeated washing since the permeability of the cells to potassium is clearly involved.

Further studies on the alkali metal requirements of ram and bull spermatozoa are presented in this paper.

II. Materials and Methods

Bull semen was collected by means of the artificial vagina, and ram semen by electrical stimulation as described by Gunn (1936). Only normal ejaculates of good motility were employed.

Suspensions were prepared by diluting 0.5-1.0 ml of semen 1 in 10, and the spermatozoa were washed by spinning this suspension four times at 1500 r.p.m. (about 300g) for 10 min.

Spermatozoa suspensions were incubated at 37°C for 4 hr. Motility was scored at hourly intervals by the system of Emmens (1947). In the analyses of variance the sum of the hourly figures has been used as unit observation and the interaction mean square as the error term.

The sodium- and potassium-fructose-phosphate diluents were isotonic and had a pH of 7.0. They were of the following composition:

Sodium diluent: 0.032M $NaH_2PO_4.H_2O$, 0.048M $Na_2HPO_4.12H_2O$, 0.040M NaCl, 0.022M fructose.

Potassium diluent: 0.032M KH_2PO_4, 0.048M K_2HPO_4, 0.040M KCl, 0.022M fructose.

Additional substances were added in concentrations indicated in the tables.

All diluents were freshly prepared each day by appropriate dilution of 0.4M stock solutions of the A.R. salts with glass-distilled water, solid fructose being added to give a concentration of 0.4 per cent (w/v).

III. Results

In view of the action of potassium in restoring the motility of washed ram and bull spermatozoa, it was of interest to compare the effectiveness of the other alkali metals. Ammonium ions were also included in these tests, as in some biological systems they are known to substitute for potassium (Boyer, Lardy, and Phillips 1942, 1943; Muntz 1947). The effects of 0.004M potassium, rubidium, caesium, lithium, and ammonia on the motility of washed ram and bull spermatozoa are given in Table 1. Four ejaculates were used with each.

The results were subjected to analysis of variance and the results are summarized in Table 2. It is clear from the data and the analyses of variance that potassium, rubidium, and caesium considerably increased the motility score.

Lithium, on the other hand, was toxic whilst ammonia had no significant effect. Rubidium was as good as, or better than, potassium but caesium was not as effective. In general, ram and bull spermatozoa reacted similarly.

Table 1

EFFECT OF 0·004M ALKALI METAL AND AMMONIUM IONS ON THE TOTAL MOTILITY SCORE OF RAM AND BULL SPERMATOZOA WASHED FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT

Species	Ejaculate	Ion					
		A Nil	*B* Lithium (LiCl)	*C* Potassium (KCl)	*D* Rubidium (RbCl)	*E* Caesium (CsCl)	*F* Ammonium (NH_4OH)
Ram	1	8·00	5·50	15·00	14·50	8·25	5·75
	2	14·25	10·00	20·00	20·00	17·25	12·75
	3	5·50	3·75	15·25	15·50	10·00	7·25
	4	6·50	4·50	16·00	17·25	8·25	5·50
	Mean	8·50	6·50	16·50	16·75	11·00	7·75
Bull	1	9·00	0·50	14·50	14·75	14·25	9·00
	2	4·75	1·50	9·25	9·75	6·75	10·25
	3	5·25	1·50	8·75	9·00	5·75	3·25
	4	6·75	1·25	8·75	10·00	7·50	5·75
	Mean	6·50	1·25	10·25	11·50	8·50	7·00

Table 2

ANALYSIS OF VARIANCE FOR THE DATA IN TABLE 1

Source of Variation	Degrees of Freedom		Variance Ratios			
			Ram		Bull	
Between treatments	5		54·5**		13·4**	
Effect of lithium (*A v. B*)		1		17·6**		29·9**
Effect of potassium (*A v. C*)		1		163·8**		16·3**
Effect of rubidium (*A v. D*)		1		174·2**		21·4**
Effect of caesium (*A v. E*)		1		14·4**		4·9*
Effect of ammonium (*A v. F*)		1		1·5		0·4
Between ejaculates	3		36·2		6·8	
Interaction (error)	15		25		59	

* $P<0{\cdot}05$.

** $P<0{\cdot}01$.

The control diluent used in the previous experiment for demonstrating the effect of potassium and the rare alkali metals contained sodium as the only cation. To test whether sodium itself was important in the functioning of mammalian spermatozoa, motility comparisons were made between spermatozoa washed in:

TABLE 3

EFFECT OF 0·004M NaCl ON TOTAL MOTILITY SCORE OF SPERMATOZOA WASHED FOUR TIMES IN POTASSIUM-FRUCTOSE-PHOSPHATE DILUENT, AND COMPARISON OF THIS DILUENT WITH ONE HAVING Na^+ AS SOLE CATION

Species	Ejaculate	Treatment			
		A. Washed in Potassium Diluent	*B*. Washed in Potassium Diluent+0·004M NaCl	*C*. Unwashed in Sodium Diluent	*D*. Unwashed in Potassium Diluent
Ram	1	7·25	8·00	9·75	3·25
	2	9·25	9·00	16·25	6·50
	3	13·75	13·75	18·75	11·25
	4	13·25	13·75	15·50	10·00
	Mean	11·00	11·25	15·00	7·75
Bull	1	4·00	5·75	17·75	9·25
	2	4·50	6·25	20·00	9·25
	3	3·75	4·00	14·75	3·75
	4	4·50	5·25	19·50	5·50
	Mean	4·25	5·25	18·00	7·00

TABLE 4

ANALYSIS OF VARIANCE FOR THE DATA IN TABLE 3

Source of Variation	Degrees of Freedom		Variance Ratios			
			Ram		Bull	
Between treatments	3		25·0**		86·3**	
A v. B		1		0·1		1·4
C v. D		1		74·4**		130·5**
(*A*+*B*) *v.* (*C*+*D*)		1		0·5		127·1**
Between ejaculates	3		29·5**		4·6*	
Interaction	9		23		30	

* $P<0{\cdot}05$.

** $P<0{\cdot}01$.

(*a*) A sodium-free diluent in which potassium was the only cation; (*b*) The same diluent with 0.004M NaCl added. Motility observations were simultaneously made on unwashed spermatozoa suspended in solutions containing (*c*) sodium and (*d*) potassium as sole cations, to see which was the more effective diluent.

Table 5

TOTAL MOTILITY SCORES OF SPERMATOZOA WASHED FOUR TIMES IN SODIUM-FRUCTOSE-PHOSPHATE DILUENT, 0·01M ACETYLCHOLINE AND 10^{-5}M PHYSOSTIGMINE ADDED SINGLY AND IN COMBINATION (EFFECT OF 0·004M KCl FOR COMPARISON)

Species	Ejaculate	Substances Added				
		A. Nil	*B*. Acetylcholine	*C*. Physostigmine	*D*. Acetylcholine + Physostigmine	*E*. Potassium Chloride
Ram	1	17·50	16·00	18·50	16·75	20·00
	2	10·50	9·50	10·00	9·25	16·25
	3	14·00	11·50	19·00	15·75	20·00
	4	5·25	4·25	5·50	5·25	17·50
	Mean	11·75	10·25	13·25	11·75	18·50
Bull	1	4·50	3·00	3·25	2·75	5·50
	2	3·75	5·75	5·25	3·50	5·50
	3	3·25	3·00	2·25	6·50	10·75
	4	3·25	3·00	1·25	4·25	7·00
	Mean	3·25	3·75	3·00	4·25	7·25

Table 6

ANALYSIS OF VARIANCE FOR THE DATA IN TABLE 5

Source of Variation	Degrees of Freedom		Variance Ratios			
			Ram		Bull	
Between treatments	4		8·2**		3·8*	
A v. B		1		0·9		0·1
A v. C		1		0·9		0·0
A v. D		1		0·0		0·7
A v. E		1		8·0**		10·3**
Between ejaculates	3		22·3**		1·1	
Interaction	12		78		48	

* $P<0{\cdot}05$.

** $P<0{\cdot}01$.

The results of tests on four ram and four bull ejaculates are set out in Table 3, and the analyses of variance are summarized in Table 4. The motility of unwashed ram and bull spermatozoa was better maintained in a diluent with the sole cation sodium than with potassium. However, the addition of

0.004M NaCl to the sodium-free diluent did not significantly increase the motility score of the washed spermatozoa.

Table 5 gives the results of experiments on four ram and four bull ejaculates, designed to test whether acetylcholine or physostigmine, singly or in combination, affect the motility of spermatozoa washed in a potassium-free diluent. A potassium-containing diluent was included in each experiment for comparison. The analyses of variance for both sets of data are summarized in Table 6. The beneficial effect of potassium is shown by the highly significant difference between diluents *a* and *b*. However, neither 10^{-2}M acetylcholine nor 10^{-5}M physostigmine, singly or in combination, significantly modified the motility score of either the ram or bull spermatozoa.

TABLE 7

EFFECT OF 0·004M KCl AND DESOXYCORTICOSTERONE ACETATE (1 μg/ml) ON MOTILITY OF SPERMATOZOA WASHED FOUR TIMES IN A SODIUM PHOSPHATE-FRUCTOSE DILUENT

Species	Ejaculate	Substances Added			
		A. Nil	*B.* Desoxycorticosterone Acetate	*C.* Potassium Chloride	*D.* Potassium Chloride + Desoxycorticosterone Acetate
Ram	1	7·00	6·25	15·00	9·25
	2	6·00	7·00	13·50	8·50
	3	11·00	3·00	15·00	4·25
	4	12·50	2·75	15·50	4·75
	Mean	9·25	4·75	9·25	6·75
Bull	1	7·00	2·25	12·75	3·50
	2	8·75	10·00	14·50	13·75
	3	4·00	3·25	7·75	7·00
	4	5·00	3·50	9·75	9·25
	Mean	6·25	4·75	11·25	8·50

Table 7 shows the motility scores of ram and bull spermatozoa washed four times in sodium and sodium-potassium diluents, with and without the addition of desoxycorticosterone acetate at a concentration of 1 μg/ml. These experiments, which were replicated on four ejaculates from each species, were of factorial design and were planned to demonstrate the effects of potassium and desoxycorticosterone and any interaction between them. Table 8 summarizes the analyses of variance for each species. The motility of both ram and bull spermatozoa was again much better maintained in the potassium-containing diluent ($P < 0.01$). Desoxycorticosterone, on the other hand, depressed the motility of the ram spermatozoa ($P < 0.01$) but had no significant effect on the bull spermatozoa. There was no evidence of any interaction between potassium and desoxycorticosterone in either species.

IV. Discussion

The studies of the motility of washed ram and bull spermatozoa (Table 1) show an interesting gradation in the biological properties of the alkali metals which can be readily correlated with their sequence in the periodic table. The efficiency of the alkali metals in these experiments increased with increasing atomic number, reached an optimum with potassium and rubidium, and fell off again with caesium.

Table 8

ANALYSIS OF VARIANCE FOR THE DATA IN TABLE 7

Source of Variation	Degrees of Freedom		Variance Ratios			
			Ram		Bull	
Between treatments	3		10·7**		7·3**	
Effect of potassium		1		8·2**		17·2**
Effect of desoxycorticosterone		1		22·1**		4·2**
P/D interaction		1		1·9		0·4
Between ejaculates	3		0·1		7·3**	
Residual (error)	9		112		69	

** $P<0{\cdot}01$.

Boyer, Lardy, and Phillips (1942, 1943) have shown that potassium is necessary for the conversion of phosphopyruvate to pyruvate by muscle extracts and more recently Muntz (1947) found that it also participates in the phosphokinase reaction of yeast juice. Both reactions are involved in the glycolytic cycle and might be the point at which potassium and other alkali metals affect ram and bull spermatozoa. It should be noted, however, that ammonium ions stimulated the phosphoenolpyruvate and the hexosephosphate reactions to about the same extent, whereas in the experiments reported here, ammonia had no significant effect, while potassium was highly beneficial (Tables 1 and 2). Intact spermatozoa were employed in the present tests, and it is not known with any certainty that ammonium ions are able to penetrate into these cells.

Although the experiments on washed ram and bull spermatozoa reported here and previously (Lardy and Phillips 1943; White 1953*a*, 1953*b*; Blackshaw 1953*a*, 1953*b*) clearly establish that potassium is necessary for their normal functioning, it is obvious from Table 3 that high concentrations of potassium are harmful. The adverse effect of the diluent with potassium the only cation must have been due to the toxicity of potassium rather than the absence of sodium, since the addition of 0.004M sodium chloride to the washed ram and bull spermatozoa did not significantly improve motility. It might be inferred from these experiments that sodium *per se* is not important for ram and bull spermatozoa, although the inherent toxicity of the potassium diluent may have masked any stimulating effect of the sodium ions.

The motility of ram and bull spermatozoa washed in the sodium diluent was neither improved by acetylcholine nor depressed by physostigmine (Table 5), which suggests that there is no connection between cholinesterase activity and the maintenance of potassium levels in these spermatozoa.

No studies *in vitro* seem to have been made of the effect of desoxycorticosterone on cell permeability. Observations *in vivo,* however, suggest that it decreases the permeability of the renal tubules to potassium and increases that of the tissues generally. If it increased the permeability of spermatozoa to potassium, it might be expected to decrease their motility after washing in a potassium-free diluent. Such an effect on motility is seen with ram spermatozoa in Table 7. However, the motility score was not improved by the addition of potassium to the diluent containing desoxycorticosterone, which makes it unlikely that the toxicity of the hormone was due specifically to an alteration in permeability to potassium.

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